The EMBO Journal vol.4 no.2 pp.399-406, 1985

Characterization of the prosome from *Drosophila* and its similarity to the cytoplasmic structures formed by the low molecular weight heat-shock proteins

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Communicated by P.-F.Spahr

We have identified and characterized a ribonucleoprotein structure from the cytoplasm of *Drosophila melanogaster* tissue culture cells which is equivalent to the prosome, a recently described ribonucleoprotein particle of duck and mouse cells. During the recovery period following heat shock, the low mol. wt. heat-shock proteins form cytoplasmic ribonucleoprotein particles which co-purify with the *Drosophila* prosome. Both ribonucleprotein particles share several structural properties but their protein constituents differ in their metabolism and cellular localization during the heat treatment. We also report the partial nucleotide sequences of several small RNA species associated with the *Drosophila* prosome. One of them has a strong sequence homology with the U6 mammalian small nuclear RNA.

Key words: cytoplasmic RNPs/*Drosophila* prosome/heat-shock low mol. wt. proteins/low mol. wt. cytoplasmic RNA sequence/U6 snRNA

Introduction

In *Drosophila melanogaster* cells exposed to a temperature of 37°C the low mol. wt. heat-shock proteins (low mol. wt. HSPs, characterized by mol. wts. of 22 000, 23 000, 26 000 and 27 000 daltons and abbreviated here HSP22, 23, etc.) co-purify with purified nuclei (Arrigo *et al.*, 1980; Velasquez *et al.*, 1980; Sinibaldi and Morris, 1981; Levinger and Varshavsky, 1981; Vincent and Tanguay, 1982) and display nucleic acid binding properties (Arrigo *et al.*, 1980; Kloetzel and Bautz, 1983); a high concentration of these proteins is found in nucleoli (Arrigo *et al.*, 1980). During the recovery period following the heat shock, these proteins migrate back to the cytoplasm (Arrigo *et al.*, 1980; Arrigo, 1980a, 1980b) and three of them (HSP23, 26 and 27) are detected in cytoplasmic aggregates with properties similar to those of ribonucleoprotein particles (RNPs) (Arrigo and Ahmad-Zadeh, 1981).

We have found that in cells recovering from a heat shock, the four low mol. wt. HSPs are concentrated in cytoplasmic 20S RNP(s) which co-purify with a structure normally pre-existing in the cell. This pre-existing structure has properties similar to the prosome, a recently described RNP present in the cytoplasm of duck and mouse cells (Schmid *et al.*, 1984).

Here, we report the characterization of the prosome of *Drosophila melanogaster* and a comparison of several of its properties with those of co-purifying RNP structure(s) formed by the low mol. wt. HSPs in the cytoplasm of cells recovering from a heat shock. Our results indicate that the prosome and the low mol. wt. HSPs cytoplasmic RNP(s) share many structural properties and could be related particles. However, their protein constituents differ in their metabolism and cellular localization during the heat shock. We also present the partial nucleotide sequences of several of the low mol. wt. RNA species associated with the prosome of *Drosophila* which differ from those of tRNA and 5S RNA. One of these RNA species has a strong sequence homology with mammalian U6 small nuclear RNA.

Results

An antiserum recognizing HSP23, 26 and 27

Since our experiments involve the use of a polyclonal antiserum directed against HSP23 under less stringent conditions than those previously described (Arrigo and Ahmad-Zadeh, 1981), we made a detailed characterization of this antiserum. In the presence of 1 M urea this antiserum immunoprecipitates essentially HSP23, while in less strigent conditions HSP26 and 27 are also recognized (Figure 1A). The identity of the immunoprecipitated proteins was confirmed by tryptic digest analysis (data not shown). This antiserum does not specifically immunoprecipitate the high mol. wt. HSPs (Figure 1A), nor any labelled proteins from the cytoplasm of normal cells (Figure 1B), indicating that it is specific for HSP23, 26 and 27.

Two main bands, labelled with [^{125}I]protein-A are detected by this polyclonal antiserum in immunoblots of the cytoplasmic proteins from cells recovering from a 1 h heat shock (Figure 1C). These bands reflect the recognition of HSP23 and HSP26 and HSP27, respectively. These low mol. wt. HSPs have partial sequence homology (Ingolia and Craig, 1982; Southgate *et al.*, 1983). No band is detected when the immunoblot is performed with the cytoplasmic proteins of untreated cells; however a longer exposure reveals a small amount of low mol. wt. HSPs pre-existing in normal cells (see below, Figure 2C).

Purified 20S cytoplasmic fraction isolated before, during or after a 1 h heat shock

A 20S cytoplasmic fraction containing the low mol. wt. HSPs (detected by autoradiography) and a characteristic set of Coomassie blue stained proteins has been described in cells recovering from a heat shock (see Arrigo and Ahmad-Zadeh, 1981). We have purified this particular fraction (see Materials and methods) from: (i) normal cells grown at 25°C; (ii) cells lysed immediately after 1 h of heat shock at 37°C; or (iii) cells lysed after 1 h at 37°C and 6 h at 25°C. Figure 2A shows the Coomassie blue stained proteins (mol. wt. between 20 and 30 kd and some larger) found in these 20S cytoplasmic fractions. The pattern of proteins is the same in all three cases. Thus these proteins belong to a structure which pre-exists in normal cells and remains cytoplasmic during the heat shock. These proteins are labelled with [^{35}S]methionine when the cells are at 25°C (Figure 2B, lane a) but not when the labelling is done during the heat treatment (Figure 2B, lane b); at 25°C after the heat shock their synthesis gradually

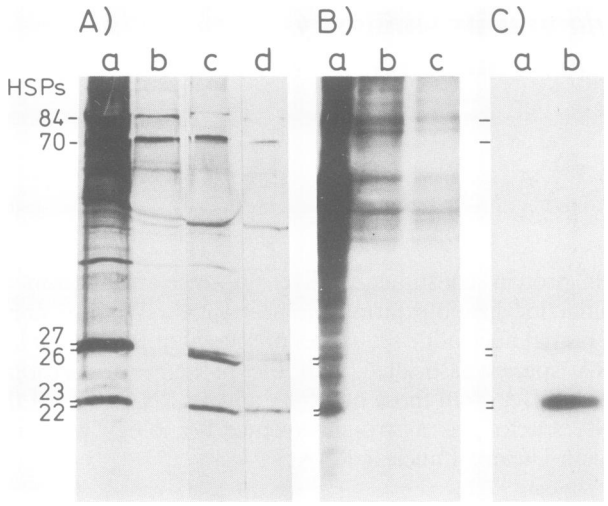

Fig. 1. Characterization of an antiserum recognizing HPS23, 26 and 27. **(A,B)** Immunoprecipitation of the [^{35}S]methionine-labelled proteins present in the 16 000 *g* cytoplasmic extracts: **(A)** cells labelled for 1 h at 37°C and further chased for 6 h at 25°C. **(B)** Cells labelled at 25°C for 1 h. Autoradiographs of the SDS-polyacrylamide gel are presented. **(a)** Aliquot of the labelled proteins present in the 16 000 *g* cytoplasmic extract. **(b,c,d)** Proteins immunoprecipitated with either non-immune **(b)** or immune anti-HSP23 serum **(c,d)** (see Materials and methods). Washings were performed either without **(b,c)** or with **(d)** 1 M urea containing buffer. **(C)** Immunoblotting experiment: the 16 000 *g* cytoplasmic supernatants isolated from normal cells or cells recovering from a 1 h heat shock were used. Autoradiographs of the immunoblots performed with anti-HSP23 serum and [^{125}I]protein-A are presented. **(a)** Normal cells, **(b)** cells allowed to recover for 6 h after a 1 h heat shock.

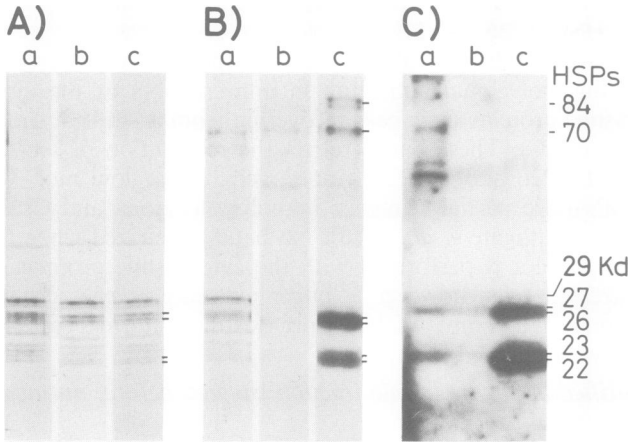

Fig. 2. Analysis of the 20S cytoplasmic fraction isolated before, during or after a heat shock. The 20S cytoplasmic fraction was isolated as described in Materials and methods before, during or after a 1 h heat shock at 37°C and its protein content was analyzed by SDS-polyacrylamide gel electrophoresis. **(A)** Coomassie blue staining; **(B)** corresponding autoradiographs. The protein constituents of the 20S cytoplasmic fraction were isolated from cells: **(a)** kept at 25°C and labelled for 1 h with [^{35}S]methionine; **(b)** immediately after 1 h of labelling at 37°C; **(c)** after 6 h of chase at 25°C following 1 h of labelling at 37°C. **(C)** Immunoblotting analysis: 20S cytoplasmic fractions purified from non-labelled cells lysed before **(a)**, during **(b)** or after the heat shock **(c)** were used. Fluorographs of the immunoblots performed with the antiserum described in Figure 1 and [^{125}I]protein A (see Materials and methods) are presented.

resumes (not shown). In addition, during the recovery period following a 1 h heat shock, pulse-chase experiments reveal the accumulation of the low mol. wt. HSPs, made during the heat treatment, in the 20S cytoplasmic fraction (Figure 2B,

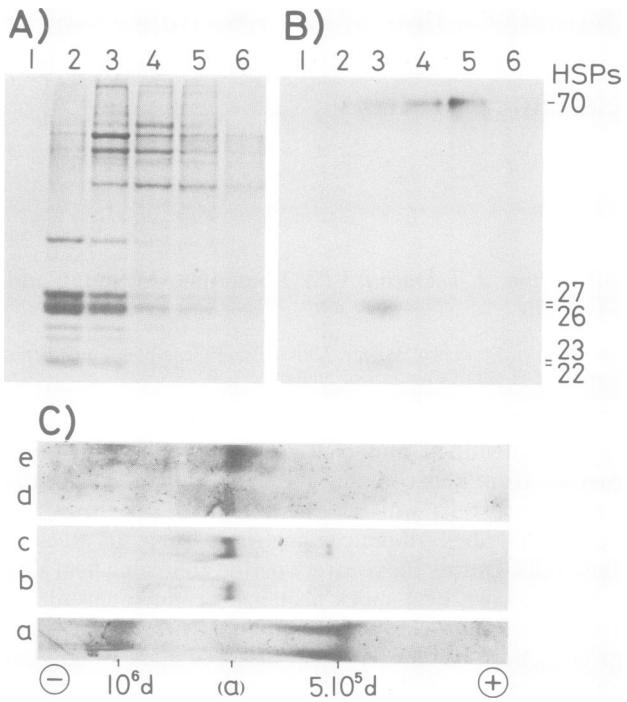

Fig. 3. Sedimentation and electrophoresis in non-denaturing conditions of the 20S cytoplasmic fraction. Cells were labelled for 1 h at 37°C with [^{35}S]-methionine and further chased for 6 h at 25°C. The 20S particle was prepared by two successive centrifugations in sucrose gradient (see Materials and methods). The proteins present in the different fractions of the second sucrose gradient were analyzed by SDS polyacrylamide gel electrophoresis. **(A)** Coomassie blue staining analysis of the proteins, **(B)** autoradiography of the gel. The top fraction of the gradient is fraction 6. A sedimentation value of 20S was calculated at the level of fraction 3. **(C)** Non-denaturing gel electrophoresis of the structure sedimenting at 20S. Tracks **(a,b,c)** Coomassie stained gels, **(d,e)** peroxidase staining of immunoblotting. **(a)** RNA polymerase subunits of *E. coli* (10^6 and 5×10^5 daltons) used as mol. wt. marker. **(b)** Analysis of the particles present in fraction 2 of the sucrose gradient described in **A**. **(c)** as **(b)** but fraction 3. **(d,e)** as **(b,c)** but immunoblotting analyses using the anti-serum described in Figure 1.

lane c; the two-dimensional gel analysis in Figure 4C shows better the four HSPs in these complexes).

To quantify the degree of accumulation of the low mol. wt. HSPs, the 20S cytoplasmic structure was isolated before, during or after the heat shock and immunoblotting experiments were performed with the antiserum described in Figure 1. The results shown in Figure 2C indicate that small but significant amounts of HSP23, 26 and 27 are detected in the 20S cytoplasmic structure purified from normal cells before any heat shock (lane a). The signal is considerably decreased in cells lysed immediately after 1 h at 37°C (lane b) and is recovered at the level of the nuclear fraction (not shown). As expected, 6 h after the heat shock the signal becomes very strong (lane c). A weak signal from a 29-kd protein remains constant (this may reflect a small cross-reactivity of the antiserum with a protein of the 20S pre-existing cytoplasmic structure) and provides an internal control. The low mol. wt. HPS pre-existing in the 20S cytoplasmic structures of normal cells represent $<10\%$ of the total amount accumulated within 6 h after 1 h of heat shock.

These results indicate that after the heat shock the low mol. wt. HSPs co-purify with a normal cytoplasmic constituent of the cell, the proteins of which have a different metabolism and cellular localization during the heat treatment.

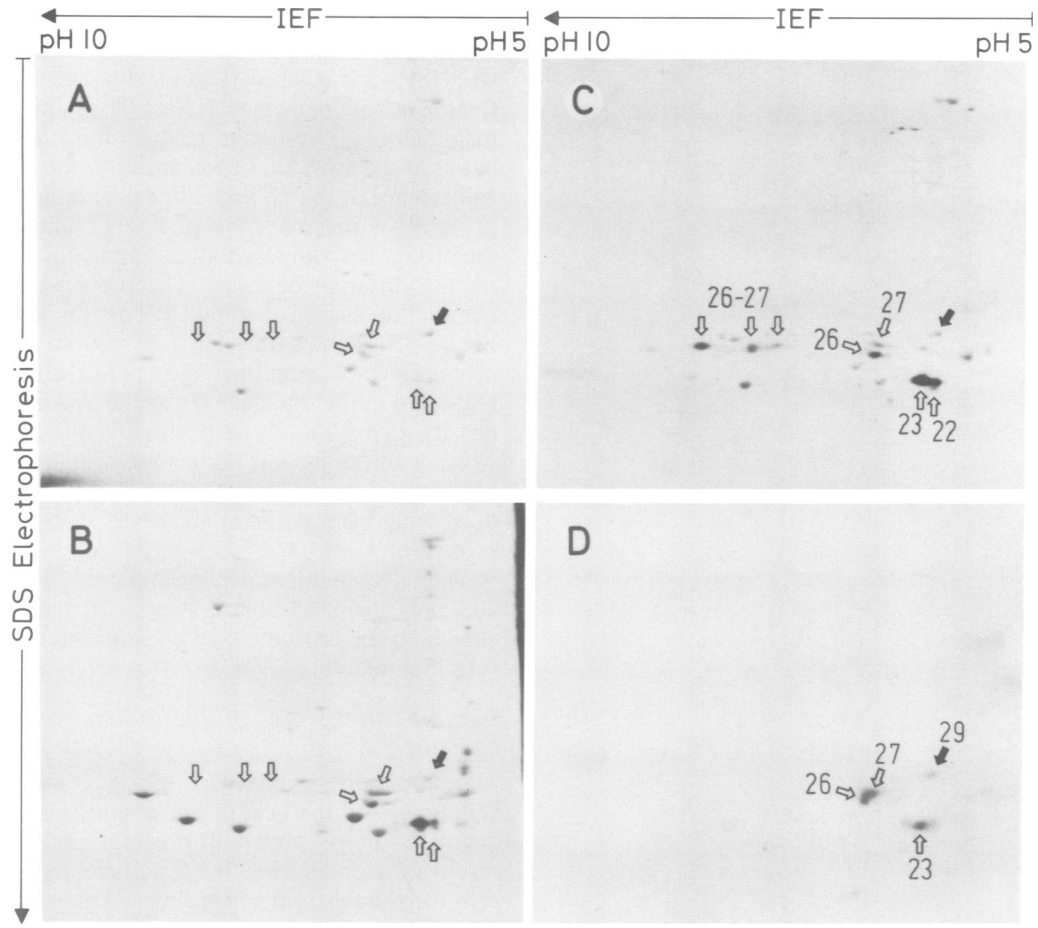

Fig. 4. Two-dimensional gel analysis of the protein constituents of the purified 20S cytoplasmic particles. The 20S cytoplasmic fraction was analyzed in two-dimensional gels as described in Materials and methods. **(A)** Coomassie blue staining of the proteins present in the 20S fraction isolated after the heat shock, a similar protein pattern is observed before or during the heat shock. **(B)** Fluorography, the proteins were isolated from cells labelled for 1 h at 25°C with [^{35}S]methionine. **(C)** Same as **(B)** but the labelling was for 1 h at 37°C and followed by a chase of 6 h at 25°C. **(D)** Immunoblotting (the cells were heat shocked for 1 h at 37°C and allowed to recover for 6 h at 25°C without any labelling). The antiserum described in Figure 1 and [^{125}I]protein-A were used as described in Materials and methods. A fluorograph of the nitrocellulose filter is presented. Open arrows indicate the positions of the low mol. wt. HSPs. The black arrow points to the 29-kd protein. IEF: isoelectrofocusing.

Sucrose gradient centrifugation and non-denaturing gel electrophoresis of the 20S cytoplasmic fraction

Figure 3A and B shows an analysis of the proteins present in the different fractions of a sucrose gradient sedimentation of the 20S cytoplasmic particles from cells allowed to recover for 6 h after 1 h heat shock. Only the slower sedimenting fraction of the 20–30 kd Coomassie blue stained proteins belonging to the 20S pre-existing structure co-sediment with the labelled low mol. wt. HSPs. This sedimentation pattern is unaffected by the presence of either 10 mM EDTA or 0.5 M NaCl or by prior treatment with pancreatic RNase in the presence of magnesium (data not shown). The two fractions of the sucrose gradient containing the 20S particles have been further analyzed by non-denaturing gel electrophoresis (Figure 3C). Both fractions contain a Coomassie blue stained complex [band (a)] which migrates with an apparent mol. wt. of $\sim 7 \times 10^5$ daltons. Immunoblotting experiments (Figure 3C, d and e) using the anti-HPS23, 26 and 27 serum show that these HSPs belong to structures displaying a similar electrophoretic migration as band (a). An analysis of these complexes in a second dimension in the presence of SDS reveals the 20–30 kd stained proteins as well as the labelled low mol. wt. HSPs (not shown). The 20S pre-existing cytoplasmic structure and the low mol. wt. HSPs particle(s) thus differ slightly in their sedimentation pattern but have a similar electrophoretic mobility in non-denaturing gels.

Two-dimensional gel electrophoresis of the proteins from the 20S cytoplasmic fraction

Figure 4 shows a two-dimensional gel electrophoregram of the proteins from a purified 20S cytoplasmic fraction. At least 20 different Coomassie blue stained proteins can be detected; neither the complexity nor the intensity of this protein pattern changes when the fraction is purified before, during or after a heat shock (not shown). The analysis of autoradiographs and immunoblots indicates that the low mol. wt. HSPs have a size and isoelectric value similar to some of the protein constituents of the pre-existing structure, except for the two basic polypeptides (Figure 4C) supposed to be modified forms of HSP26 and 27 (Buzin and Pedersen, 1981). Note that the antiserum recognizes HSP23 and only the acidic forms of HSP26 and 27, while HSP22 is not recognized although it is present in the 20S particles isolated from cells recovering from a heat shock. An additional spot (black arrow, Figure 4) corresponding to the 29-kd protein (described in Figure 2C) and belonging to the 20S pre-existing structure is also slightly recognized. If the RNase treatments performed before the two-dimensional gel analysis (see Materials and methods) are omitted, the low mol. wt. HSPs, as well as the proteins of the

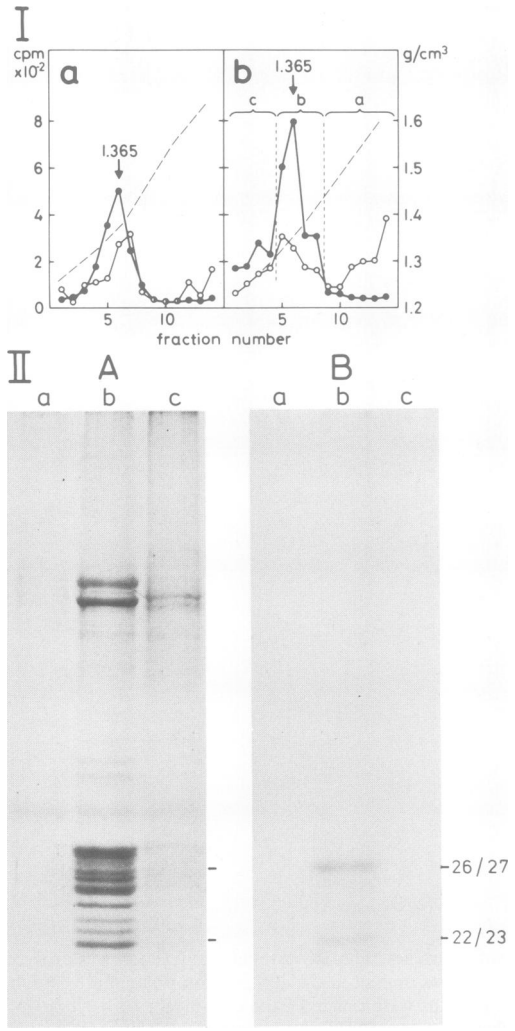

Fig. 5. CsCl density gradient analysis of the 20S cytoplasmic particles. **(I)** CsCl density gradient analysis of the cytoplasmic 20S particles. The 20S particles were isolated from u.v.-irradiated cells and analyzed in CsCl density gradient as described in Materials and methods. Gradients were collected from the bottom and aliquots of each fraction were used to determine the density and the TCA insoluble counts. Cells were labelled with [^{35}S]-methionine and [^{3}H]uridine for **(a)** 1 h at 25°C or **(b)** 1 h at 37°C and allowed to recover for a 6 h chase period at 25°C. ●—●: TCA insoluble ^{35}S c.p.m.; ○—○: TCA insoluble ^{3}H c.p.m. **(II)** SDS-polyacrylamide gel analysis of the proteins present in the different pools of the CsCl density gradient shown in panel **Ib**. **(A)** Coomassie blue staining. **(B)** Autoradiography. **(a)** Pooled fractions with a density >1.45 g/cm³. **(b)** Fractions of density from 1.33 to 1.45 g/cm³. **(c)** Fraction lighter than 1.33 g/cm³.

pre-existing 20S structure, do not focus, as with other RNA binding proteins (O'Farell, 1975; Khandjian *et al.*, 1982; Unteregger *et al.*, 1983). A similar observation has been made concerning HSP68 and 70 (Storti *et al.*, 1980; DiDomenico *et al.*, 1982).

The pre-existing 20S structure and the low mol. wt. HSPs complex(es) are ribonucleoprotein particles

To compare the density of the pre-existing structure with that of the low mol. wt. HSPs particle(s), cells were labelled with [^{35}S]methionine and [^{3}H]uridine for, respectively, 1 h at 25°C or 1 h at 37°C with a chase of 6 h at 25°C. Cross-linking was performed *in vivo* by u.v. irradiation of the cells and the fixed particles were analyzed in CsCl density gradients (see Materials and methods). The ^{35}S-labelled material from normal cells bands with a density of 1.365 g/cm³ (Figure 5Ia), consistent with an RNA content of ~20% (Spirin, 1969). A similar peak of density is observed when only the low mol. wt. HSPs are labelled (Figure 5Ib) and it is unaffected by treatment with pancreatic RNase (20 μg/ml in the presence of magnesium). If u.v. light irradiation is omitted, the ^{35}S-label bands at a density of 1.31 g/cm³ (not shown). Note the poor coincidence of the ^{35}S and ^{3}H peak and the different densities of the RNA made at 25°C and 37°C. These complexes may, therefore, be composed of a family of sub-particles with slightly different densities. The protein content of the 20S material further purified by CsCl density gradient is shown in Figure 5II. The characteristic 20–30 kd Coomassie stained proteins, belonging to the pre-existing structure, are recovered at the same density as the low mol. wt. HSPs. The density of the 20–30 kd proteins is the same irrespective of whether the analysis is performed with 20S material purified before, during or following a 1 h heat shock. These results indicate that both particles are RNPs with a ratio of RNA to protein of ~1 to 4.

The sedimentation, density and protein composition of the 20S pre-existing structure suggest that this particle is very similar to the prosome of the cytoplasm of duck and mouse cells (Schmid *et al.*, 1984).

Electron microscopy of the 20S pre-existing cytoplasmic structure

The 20S particles have a highly organized morphology (Figure 6) and the field at low magnification shows the high degree of purity and uniformity of these particles. Similar results were obtained with 20S particles from normal cells and cells recovering from a heat shock. This suggests that the particles detected by electron microscopy correspond to the 20S pre-existing structures described above. Two types of particles are seen in Figure 6: (i) one round, raspberry-shaped, with a diameter of ~12 nm and a dense central zone (not stained with uranyl acetate), although some appear without this central zone, probably because of their orientation. (ii) A second, cylindrical in shape, is formed of a stack of four subunits with the same diameter as the round particles, their length is ~17 nm. The morphology and the size of the 20S pre-existing structure are identical to those of the avian erythroblast prosomal structure described recently by Schmid *et al.* (1984). A mol. wt. of ~4.5 x 10^5 daltons can be calculated for the raspberry-shaped particle using the dimensions and the density determined above. However, this is an underestimate because the volume decreases on air drying highly hydrated particles (see for example Vasiliev, 1971).

RNA analysis of the 20S cytoplasmic RNP particles

The 20S RNP particles were isolated from cells labelled *in vivo* with [^{32}P]orthophosphate for 4 h at 25°C and their RNA was purified and analyzed in polyacrylamide gels in the presence of 7 M urea (Figure 7A). Autoradiographs of the gel revealed two major RNA bands with similar migration to tRNAs. In addition, prolonged exposure revealed several other RNA species (between 50 and 120 nucleotides in size). When a 1 h heat shock was included at the beginning of the labelling period, no major differences were seen in the labelled pattern of the RNAs. These 4S RNAs are present in the 20S RNPs (7 x 10^5 daltons) further purified by means of non-denaturing gel electrophoresis as described in Figure 3C (not shown). This makes it unlikely that they are free tRNAs contaminating the preparations. Our analysis in one dimension indicates that, as in the case of duck erythroblasts and mouse

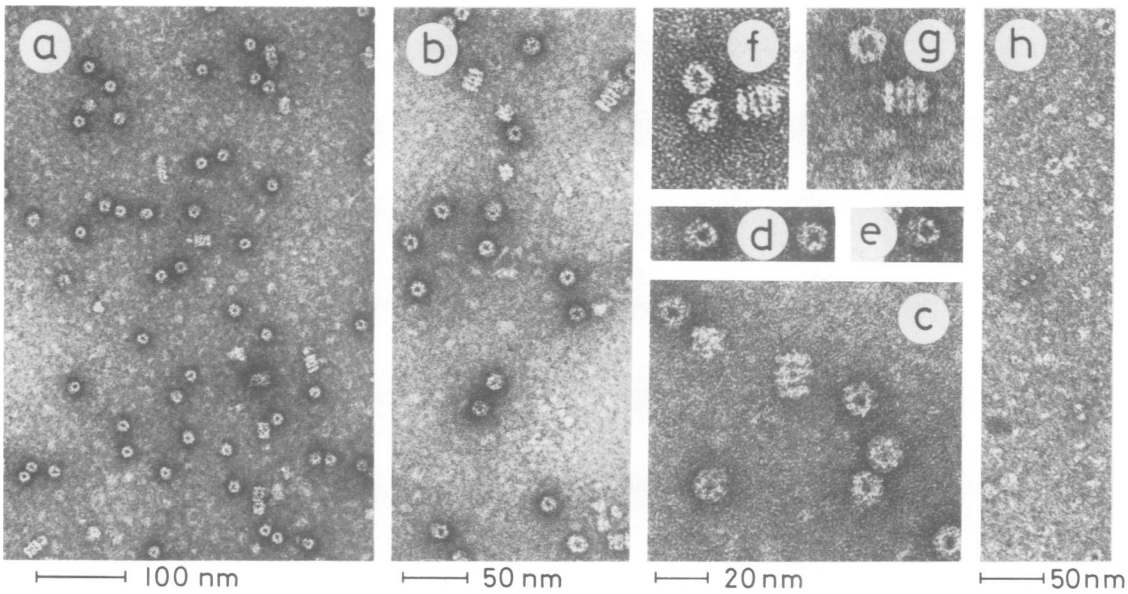

Fig. 6. Structural characterization of the purified 20S RNP particles by electron microscopy (**a**–**h**): electron micrographs of the purified 20S RNP particles from sucrose gradient fractions. (**a**) Low magnification view showing the two types of particles (rounded and cylindrical) (Bar = 100 nm). (**b**) Intermediate magnification (Bar = 50 nm). (**c**–**g**) Higher magnification showing the different morphologies (Bar = 20 nm). A circular (**c,d**), X shaped (**g,d,e**) or linear depression (**f,c**) can be seen within the rounded raspberry-like particles. Some present no depression (**c**). Cylindrical particles present four subunits (**f,g,c**) and could be stacks of the rounded particles described above. (**h**) Control experiment showing the morphology of the material sedimenting at ~10S in the last step of purification of the 20S particles. Very few rounded particles are seen and present no ring of staining by uranyl acetate (Bar = 50 nm).

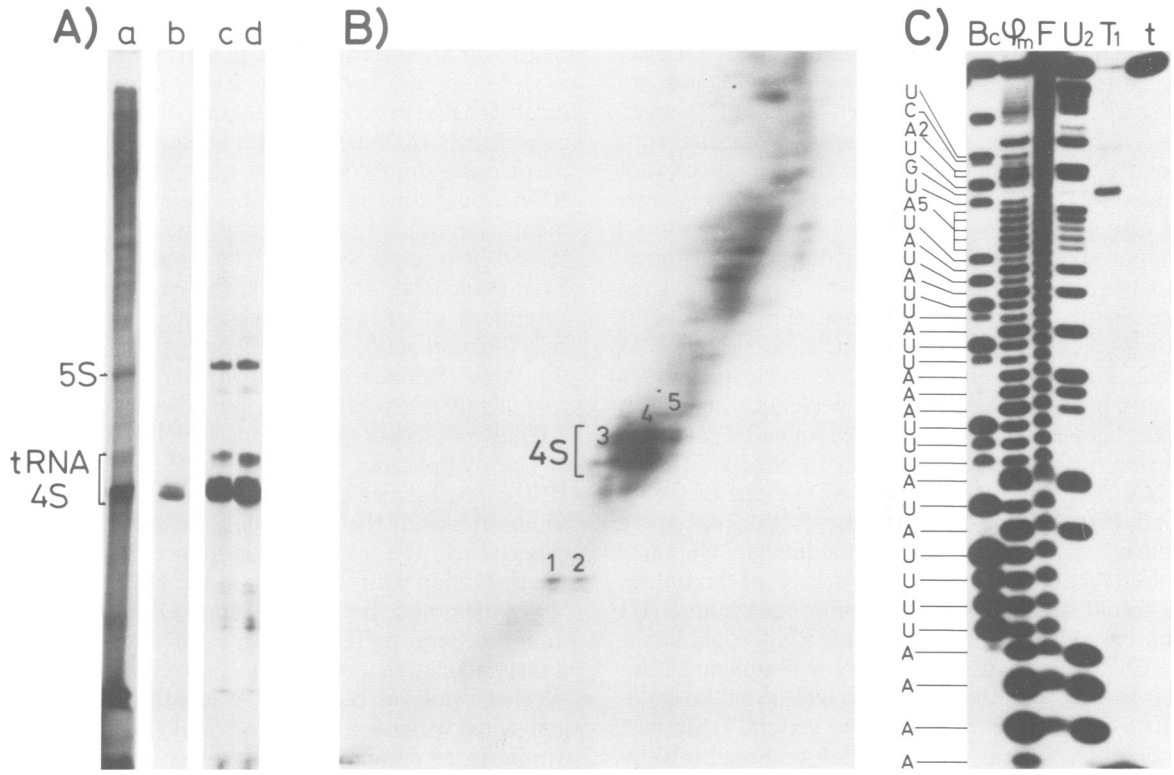

Fig. 7. Analysis of the RNA associated with the purified 20S cytoplasmic RNP particles. (**A**) RNA labelled *in vivo* with [^{32}P]orthophosphate. The 20S RNPs from normal cells (prosome) was purified from cells labelled *in vivo* for 4 h with [^{32}P]orthophosphate as described in Materials and methods. The labelled RNA was analyzed in 7 M urea, 8% polyacrylamide gels. (**a**) tRNA and 5S RNA from *in vivo* labelled total polysome RNA as mol. wt. marker. (**b,c**) *In vivo* labelled RNA from the 20S RNPs, gels were autoradiographed at −70°C using a screen for 18 h (**b**) or for 1 week (**c**). (**d**) as (**c**) but the 20S RNP was purified from cells labelled for 1 h at 37°C followed by 3 h at 25°C. (**B**) Two-dimensional gel electrophoresis of the *in vitro* 5′ end-labelled RNA. The first direction of migration is from left to right in 8% polyacrylamide 6 M urea pH 3.5, the second one is from bottom to top in 12% polyacrylamide at pH 8.3. The RNA, purified from the 20S RNP complex isolated 6 h after the heat shock, was labelled *in vitro* with [γ-^{32}P]ATP and T4 kinase and analyzed by two-dimensional gel electrophoresis as described in Materials and methods; autoradiography was for 30 min. Spots 1–5 have been eluted, purified and sequenced (see Figure 8). (**c**) RNA sequencing gel analysis (RNA spot 5 from **B**). t,T1,U2,F,ψm and Bc refer to control, T1 RNase, U2 RNase, formamide, ψm RNase and *Bacillus aureus* RNase digestion, respectively. Autoradiography was for 3 days at −70°C using a screen.

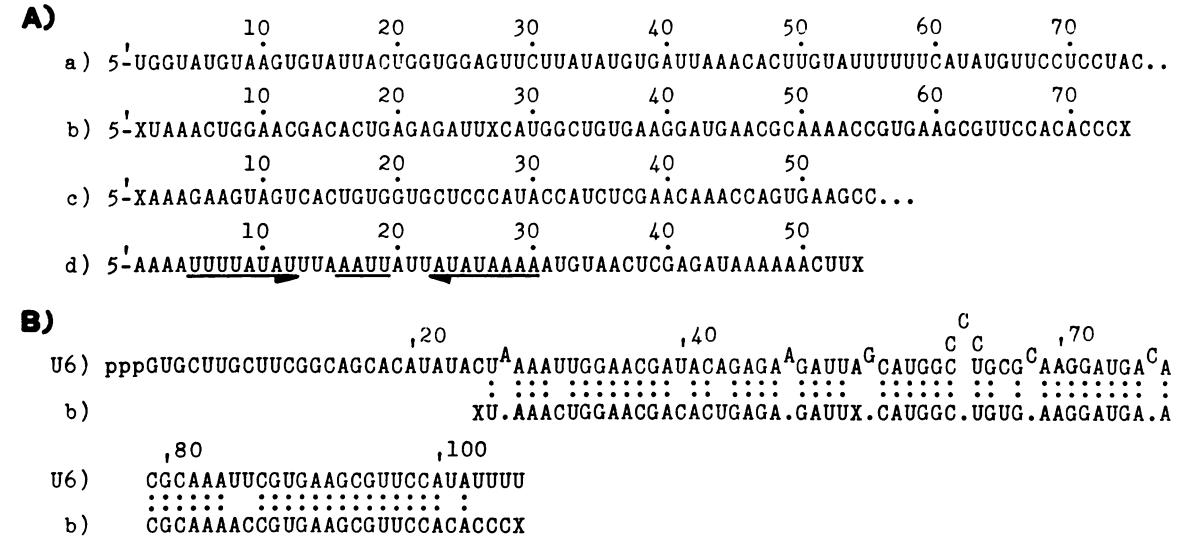

Fig. 8. RNA sequences of four small cytoplasmic RNAs associated with the 20S RNP particles. (**A**) The sequences of RNAs a,b,c,d correspond respectively to the RNA spots 1–2,3,4,5 shown in Figure 7B. The RNA in (a) corresponding to the RNA spots 1 and 2 of Figure 7B is 110 nucleotides long and only the first 75 nucleotides are presented. RNA in (c) is 60 nucleotides long and only the 55 first nucleotides are shown. The other two RNAs (b,d) are completely sequenced. Note the underlined complementary sequences (position 5–30) in (d). (**B**) Sequence homologies detected by computer analysis (see Materials and methods) between the RNA in (b) (spot 3 in Figure 7B) and U6 RNA from mouse cells (Ohshima *et al.*, 1981). A similar result is also observed with U6 RNA from rat (Harada *et al.*, 1980) or Novikov hepatoma ascites cells (Epstein *et al.*, 1980). The modified bases of U6 RNA are not represented.

erythropoietic cells (Schmid *et al.*, 1984), the *Drosophila* prosome is associated essentially with two major 4S RNAs. However, when the *Drosophila* prosomal RNA, labelled *in vitro* at its 5′ end, is purified by two-dimensional gel electrophoresis at least four to five species of 4S RNAs are detected (Figure 7B). A similar pattern of 5′ ^{32}P-labelled RNA is observed when the experiments are performed with particles isolated before, during or after the heat shock (not shown), suggesting that these RNA(s) belong to the prosome. The RNAs associated with the low mol. wt. HSPs should represent minor species compared with the prosomal ones. We have analyzed the prosomal RNAs by rapid sequencing analysis (see Figure 7B, spots 1–5); one sequencing gel is shown in Figure 7C. Of the two 110 nucleotide long RNAs (spot 1 and 2, Figure 7B) only the first 75 nucleotides from the 5′ end have been sequenced and show identical sequences (Figure 8Aa). These two identical RNA sequences contain a high proportion of U residues (45%) and are clearly different from 5S RNA. The sequence (Figure 8Ab) of one of the 4S RNAs (spot 3, Figure 7B) is clearly distinct from tRNA and a 74% homology was found with the mammalian U6 small nuclear RNA (Figure 8B). A similar degree of homology (72%) was found between the *Drosophila* and human U1 RNA (Mount and Steitz, 1981). Thus, this RNA could be the *Drosophila* U6 RNA or more probably a fragment of this RNA, as it is shorter than the known U6 RNAs and no cap is present at its 5′ end. Note the many stop codons in all these RNAs. A computer analysis (see Materials and methods) indicates that these are new species of low mol. wt. RNAs.

Discussion

We have identified and characterized a new 20S ribonucleoprotein particle from the cytoplasm of *D. melanogaster* tissue culture cells. It has a high degree of structural organisation and is composed of at least 20 different polypeptides in the 20–30 kd range and several low mol. wt. RNAs with a protein to RNA ratio of 4 to 1. The apparent mol. wt. of this particle was estimated bewteen 4.5 and 7 x 10^5 daltons, in good agreement with an RNP composed of ~20 proteins of 20–30 kd and five RNAs of 4S (mol. wt. of the RNP: 6.5 x 10^5 daltons containing 23% of RNA). The slight heterogeneity between the density of the RNA and protein constituents of these particles suggests that each individual particle may not contain the complete set of proteins and RNAs, but rather multimers of one or a subset of the constituents. Antibodies raised against the different RNP-proteins should help to resolve this. The structural properties of this *Drosophila* RNP are similar to those of the prosome from the cytoplasm of duck erythroblasts and mouse erythropoietic cells (Schmid *et al.*, 1984). The very similar size and shape of the duck (Schmid *et al.*, 1984) and *Drosophila* prosomes revealed by electron microscopy indicates that the prosome is a highly conserved cytoplasmic structure in eukaryotic cells.

The cytoplasmic structure formed by the low mol. wt. HSPs in cells recovering from a heat shock has structural similarities with the *Drosophila* prosome. Although only a subclass of the prosomal structure shows an exact cosedimentation with the low mol. wt. HSPs, their densities are very similar and they have identical electrophoretic migration in non-denaturing gels. The low mol. wt. HSP proteins have a size and charge identical to some of the prosomal proteins. We have not yet been able to identify the specific RNA(s) associated with the low mol. wt. HSPs, because this particle is a minor one compared with the prosome. We are trying to separate biochemically these two structures. Two facts allow us to conclude that the prosome and the low mol. wt. HSPs particle are not identical. (i) In contrast to the HSPs, the synthesis of the prosomal proteins is reduced during heat shock; therefore they behave like normal proteins (Mirault *et al.*, 1978). After the heat shock the reverse phenomenon is observed. (ii) The cellular localization of the proteins of both particles is different during heat-shock: at 37°C the low mol. wt. HSPs are concentrated in the nuclei (Arrigo *et al.*, 1980).

In contrast, the prosomal proteins, even those newly synthesized, remain cytoplasmic during the heat treatment. Only after the shock, it is possible to observe in the cytoplasm a co-purification of these two kinds of structure. If the prosome is composed of a family of particles containing a different set of protein and RNA constituents in multimers, the low mol. wt. HSP-RNPs could represent a particular class of these particles. Another possibility is that the low mol. wt. HSPs are associated with the prosome after the heat shock.

It is not known whether the low mol. wt. HSPs are in the form of RNP particle(s) when migrating in and out the nuclei, however once in the nuclei they behave as nucleic acid binding proteins with a high affinity for nucleoli (Arrigo, *et al.*, 1980) and hRNP particles (Kloetzel and Bautz, 1983). As early as 1979 Mitchell *et al.* proposed a function for the HSPs as RNA-binding proteins. Fractionation studies with isolated nuclei revealed that a fraction of the low mol. wt. HSPs sediments as large complexes (>100S, with a density in CsCl of 1.4 g/cm^3) together with hnRNPs; to date no traces of prosomal particles have been detected in nuclei (Arrigo, unpublished resuts).

The small amount (10%) of low mol. wt. HSPs pre-existing in the 20S RNP particles isolated from unheated cells might result from unsuspected stresses during cell culture. Since an immunofluorescence analysis of the cellular population using our antiserum reveals that only 10% of the cells grown at 25°C are positive (Duband *et al.*, in preparation) we can exclude the possibility of a basal level (10%) of the low mol. wt. HSPs in every cell.

Several small cytoplasmic RNAs (scRNAs) are found associated with the 20S RNP particles. As a roughly similar pattern of 5′ end-labelled RNA is found when the particle(s) are isolated before, during or after the heat shock we conclude that these major RNAs should belong to the prosome. It is possible that the apparent heterogeneity of the 5′ end-labelled RNAs, especially of the major 4S RNA species, is due at least in part to the phosphatase kinase step required to label the RNA. However, our RNA sequencing studies reveal that the prosome of *Drosophila* contains at least five different RNAs in the 50–80 nucleotides range and one species of 110 nucleotides. A computer analysis indicates that these small RNAs represent new species of scRNAs, with one (the 74 nucleotide long RNA) having a strong homology with the mammalian U6 small nuclear RNA (the sequence of the *Drosophila* U6 RNA is not known).

Vincent *et al.* (1983) and Schmidt *et al.* (1984) have proposed that one function of the prosome in duck and mouse cells could be at the level of a fine regulation of protein synthesis. However, once purified, no specific mRNP proteins or mRNAs were found to be associated with this particle. The investigation of the function of the prosome of *Drosophila* and by analogy the function of the structure(s) formed by the low mol. wt. HSPs will first require a biochemical separation of these two classes of particles. It is, however, already interesting to note that some of the small RNAs associated with the *Drosophila* prosome have sequences particularly rich in A and U residues and thus could be signals for the recognition of the 3′- or 5′-untranslated ends of mRNAs. On the other hand, the presence of a U6-like RNA in this particle is intriguing and may suggest a function in mechanisms involved in RNA processing.

Materials and methods

Cell cultures

The *D. melanogaster* tissue culture cell line KC161, a subline of the KC line (Echalier and Ohanessian, 1969) was provided by Dr. G.Echalier. This cell line was adapted to grow in suspension in D_{22} medium (Echalier and Ohanessian, 1970) containing 2% foetal calf serum (serum screened for viruses and mycoplasma, Gibco). The cells were grown in spinner flasks at 25°C. They had a generation time of ~15 h and were adjusted to concentrations of between 2 x 10^6 and 8 x 10^6 cells/ml.

Radiolabelling of the cells

Drosophila tissue culture cells were washed twice and concentrated 10-fold to reach ~5 x 10^7 cells/ml in D_{22} medium minus yeast extract, lactalbumin hydrolysate, serum and either methionine or phosphate. Proteins were labelled with 50 μCi/ml of [^{35}S]methionine (R.C.Amersham, 300–500 Ci/mmol) in methionine-free medium for different periods of time at 25 or 37°C with mild agitation. RNA was labelled with either 0.5 mCi/ml orthophosphate ^{32}P (R.C.Amersham, carrier free) or with 50 μCi/ml [^{3}H]uridine (R.C.Amersham, 25 Ci/mmol). The cells were then harvested and washed once in complete D_{22} medium before lysis. Chase experiments were performed at cell concentrations equivalent to those used for the culture in complete D_{22} medium containing 1 mg/ml cold methionine.

Purification of the 20S cytoplasmic structures

Cells were lysed in a solution containing 10 mM Tris-HCl, pH 7.4; 10 mM NaCl; 5 mM $MgCl_2$; 0.2% Triton X-100, 1 mM β-mercaptoethanol and 1 mM PMSF. The lysate was clarified for 10 min at 16 000 *g* and the supernatant was loaded onto sucrose gradients containing 0.5–1 M sucrose (Schwarz-Mann, RNase free) in 10 mM Tris-HCl pH 7.4; 10 mM NaCl and 5 mM $MgCl_2$. Centrifugation was for 18 h at 32 000 r.p.m. in a SB283 rotor (International centrifuge). The fractions containing the low mol. wt. HSPs were pooled and loaded on a G-150 Sephadex column equilbrated with 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and 250 mM NaCl. The excluded fractions containing the low mol. wt. HSPs were rerun in a second sucrose gradient containing 0.5 M NaCl. The fractions containing the low mol. wt. HSPs were pooled and either stored in liquid N_2 or concentrated by centrifugation for 16 h at 150 000 g in an A321 rotor (International Centrifuge). The RNP pellet was resuspended in a solution containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol and 0.2% Triton X-100 before being rerun in a third sucrose gradient.

CsCl density gradient analysis of the 20S cytoplasmic structures

Analysis of the 20S cytoplasmic structures in CsCl density gradients was carried out either directly or after *in vivo* u.v. light cross-link and further isolation of the 20S cytoplasmic structures. U.v. light cross-linking of the proteins to nucleic acids was performed *in vivo* on intact cells as previously described (Arrigo *et al.*, 1983) with some modifications: cells were pelleted and resuspended in a 100 mm Petri dish using 1 ml of D_{22} medium and were irradiated with u.v. light (15 W, germicidal lamp, 252 nm) for 5 min at 5°C and at a distance of 4 cm from the lamp (Wagenmakers *et al.*, 1980). The cells were then lysed and the 20S cytoplasmic structures isolated as already described. Solid CsCl was added to the different preparations (fixed or not) in order to reach a density of 1.3 g/cm^3 in a final volume of 5 ml. These solutions were layered over 5 ml solutions made of the same buffer but of a density of 1.5 g/cm^3 in CsCl. Centrifugation was for 65 h at 33 000 r.p.m. (140 000 *g*) in a SW40 Beckman rotor at 18°C. Fractions were collected from the bottom and aliquots were used to determine the density from the refractory index and the radioactivity by liquid scintillation spectrometry after cold TCA (30% final volume) precipitation of the samples. To analyze the protein content of some defined fractions, the CsCl was removed by dialysis and the samples were treated with pancreatic RNase (10 μg/ml, 30 min at 37°C). The proteins were precipitated with cold TCA before being analyzed in SDS gels.

Non-denaturing gel electrophoresis of the 20S cytoplasmic structures

This was carried out in 2% acrylamide, 0.5% agarose gels in Tris-borate buffer, pH 8.3 as described by Dahlberg *et al.* (1969). Samples from the sucrose gradients were analyzed directly (without boiling) in the presence of 20 mM EDTA and 0.1% bromophenol blue. After a pre-electrophoresis of 20 min at 4°C and 200 V, the samples were applied to the gel and electrophoresed for 150 min at the same voltage.

Immunoprecipitation of proteins

Anti-HSP23 serum was obtained as already described (Arrigo and Ahmad-Zadeh, 1981). For immunoprecipitation, [^{35}S]methionine-labelled cells were lysed and the cytoplasmic fraction containing the HSPs was incubated with 15 μl of either immune or non-immune sera for 2 h at 4°C. 100 μl of protein-

A Sepharose (Pharmacia) was added and the incubation was continued for an additional 30 min at 4°C. Washing was performed three times either with a solution containing: 50 mM Tris-HCl, pH 7.5; 1 mM $MgCl_2$ and 0.1% Nonidet P-40 or with a solution containing 50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 20 mM EDTA; 1 M urea; 1% Triton X-100; 1% deoxycholate (Sigma) (solution RIPA-urea). Samples were resuspended in SDS gel sample buffer (0.07 M Tris-HCl, pH 6.8; 11% glycerol; 0.003% bromophrenol blue; 3% SDS; 5% β-mercaptoethanol), heated to 100°C for 1 min and centrifuged 2 min in an Eppendorf centrifuge. The supernatant was analyzed on SDS gels.

Immunoblotting

Immunoblotting was performed essentially as described by Bowen *et al.* (1980) using the electrophoretic transfer described by Zeller *et al.* (1983). Anti-HSP23 serum was diluted 30 times and the detection was performed with 10 μCi/ml of [^{125}I]protein-A (R.C.Amersham, 37 mCi/mg). Autoradiography of the nitrocellulose sheets was done at −70°C on Fuji X-ray film with the aid of Ilford fast tungstate intensifying screens. The detection was also done with the peroxidase method (Towbin *et al.*, 1979).

Polyacrylamide gel analysis of proteins

Proteins were precipitated with 20% cold TCA (final concentration) for 3 h at 0°C. Proteins were washed with acetone, resuspended in gel sample buffer and heated to 100°C for 1 min. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (12% acrylamide, 1.3% bis-acrylamide) as described by Laemmli (1970). Gels were fixed, stained with Coomassie blue, destained and photographed. Proteins were detected by autoradiography or fluorography (Laskey and Mills, 1975; Chamberlin, 1979) on Fuji X-ray film.

Two-dimensional gel electrophoresis of proteins

Two-dimensional gel electrophoresis was performed according to O'Farell (1975) as modified by Khandjian and Türler (1983). RNPs pelleted for 16 h at 150 000 *g* were resuspended in 20 μl of 10 mM Tris-HCl, pH 7.4, containing 100 μg of pancreatic RNase and 10 U of T1 RNase and incubated for 30 min at room temperature. Samples were then mixed with the urea containing lysis buffer and loaded at the anodic end of the first dimension gel containing 2% w/v ampholytes, pH range 3.5 – 10 (LKB-Produkter AB, Bromma, Sweden). Electrofocusing was for 5000 V/h. After SDS gel electrophoresis in the second dimension, proteins were revealed as mentioned above.

Polyacrylamide gel and sequence analyses of RNA

^{32}P-Labelled 20S RNPs were digested for 1 h at 37°C with 1 mg/ml proteinase K in the presence of 0.1% SDS. The labelled RNAs were extracted with phenol-chloroform, precipitated by ethanol and analyzed in 12% polyacrylamide gel containing 7 M urea (Darlix *et al.*, 1979). For sequencing, the RNAs were dephosphorylated using purified calf intestine phosphatase and 5′ ^{32}P-labelled in the presence of [α-^{32}P]ATP and T4 polynucleotide kinase (Darlix *et al.*, 1979). The labelled RNAs were then resolved by two-dimensional polyacrylamide gel electrophoresis: 8% (w/v) acrylamide, 7 M urea, 25 mM citric acid (1st); 12% (w/v) acrylamide, 90 mM Tris-borate (pH 8.3) (2nd). Spots of 5′ ^{32}P-labelled RNAs were cut out, eluted and purified further on a sequencing polyacrylamide gel in 8 M urea (Maxam and Gilbert, 1977; Darlix and Spahr, 1983). The purified 5′ ^{32}P-labelled small RNAs were sequenced following the method described by Donis-Keller *et al.* (1977) and Simonscists *et al.* (1977).

Electron microscopy

A drop of the sucrose gradient fraction containing the purified 20S RNP particles was loaded onto freshly glow-discharged grids coated with carbon, the excess of liquid was removed with filter paper and the grids were rinsed with double distilled water. Staining was performed with 0.1% uranyl acetate, the excess liquid was removed and the grids were allowed to dry in air. Microphotographs were taken with a Phillips 300 electron microscope at 80 kv. The sizes of the particles were measured on 400 000 x enlarged prints.

Computer analysis

RNA sequences were compared to a DNA sequence bank (GENBANK, 3 x 10^6 bp). The WORDSEARCH program (Devereux and Haeberli, 1984) was used on a Digital Equipment Corporation (DEC) Vax 11/750 computer.

Acknowledgements

We wish to thank O.Jenni and Y.Epprecht for drawings and plates, C.Tonka for cell culture, E.Boy de la Tour for his help in the electron microscopy analysis, BIOGEN company for computing facilities, C.Meric for helpful discussions and F.Mulhauser for excellent technical assistance. This research was suppoted by a grant from the Swiss National Science Foundation No. 3.321.82. One of us (E.W.K.) was supported by a Hoffmann-La Roche fellowship and by the Swiss National Science Foundation (grant No. 3.567.83 to R.Weil).

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Received on 4 October 1984; revised on 27 November 1984