Co-purification of a small RNA species with multicatalytic proteinase (proteasome) from rat liver

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Previous studies have come to different conclusions about the presence of RNA in particles known variously as prosomes, proteasomes or multicatalytic proteinase (MCP). To determine the reason for this, MCP was isolated from rat liver by 4 different purification protocols. One major band of RNA, about 80 nucleotides in length, co-purified in all preparations. The amount of RNA detected was less than one molecule per MCP particle suggesting that there may be more than one population of MCP in rat liver cells.

Multicatalytic proteinase; Proteasome; RNA

1. INTRODUCTION

The multicatalytic proteinase (MCP) or proteasome is a high molecular mass (700 000 Da) complex with a cylindrical structure composed of a characteristic set of proteins [1]. Those authors investigating the particle for its endopeptidase activity have, in general, named the particle the multicatalytic proteinase complex due to its possession of different types of activities which can be differentially inhibited and activated [2]. Those authors isolating the particle as a ribonucleoprotein (RNP) associated with mRNA repression have named it a prosome [3]. It is now assumed, from comparisons of the proteolytic activity and immunological cross-reactivity of MCP and prosome preparations that these particles are the same [4,5], although they are not necessarily homologous. Kloetzel et al. [6] separated 19 S scRNPs from *Drosophila* into 3 subpopulations by ion exchange chromatography. Scherrer et al. [7] concluded that prosomes are a biophysically quite homogeneous population of biochemically heterogeneous RNP particles.

Although it is generally agreed that prosomes contain RNA [3, 8-11] there is disagreement concerning the existence of RNA in MCP [12] and other cylindrical particles [13-15]. These contradictory observations may possibly have arisen from the use of very different protein purification procedures, and, in some cases, by the use of insensitive RNA detection methods. For example, prosomes are usually isolated by sucrose gradient centrifugation methods whereas MCP and other similar particles have been purified by a variety of procedures involving several chromatographic steps. In studies where RNA has been detected, radiolabeling techniques

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were employed, while negative conclusions were frequently drawn from less sensitive RNA detection methods, such as the Orcinol reaction [12], the ratio of absorbance at 280 nm and 260 nm [11] and ethidium bromide staining of RNA loaded on to polyacrylamide gels [13].

The discrepancy over the RNA content of MCP needs to be resolved before any further progress on the composition and structure of this complex can be made. For this reason we decided to investigate the RNA content of MCP from rat liver and determine if the detection of RNA depended upon the method of purification. MCP was purified by 4 different methods and the presence of RNA investigated by radiolabeling of the 3' end.
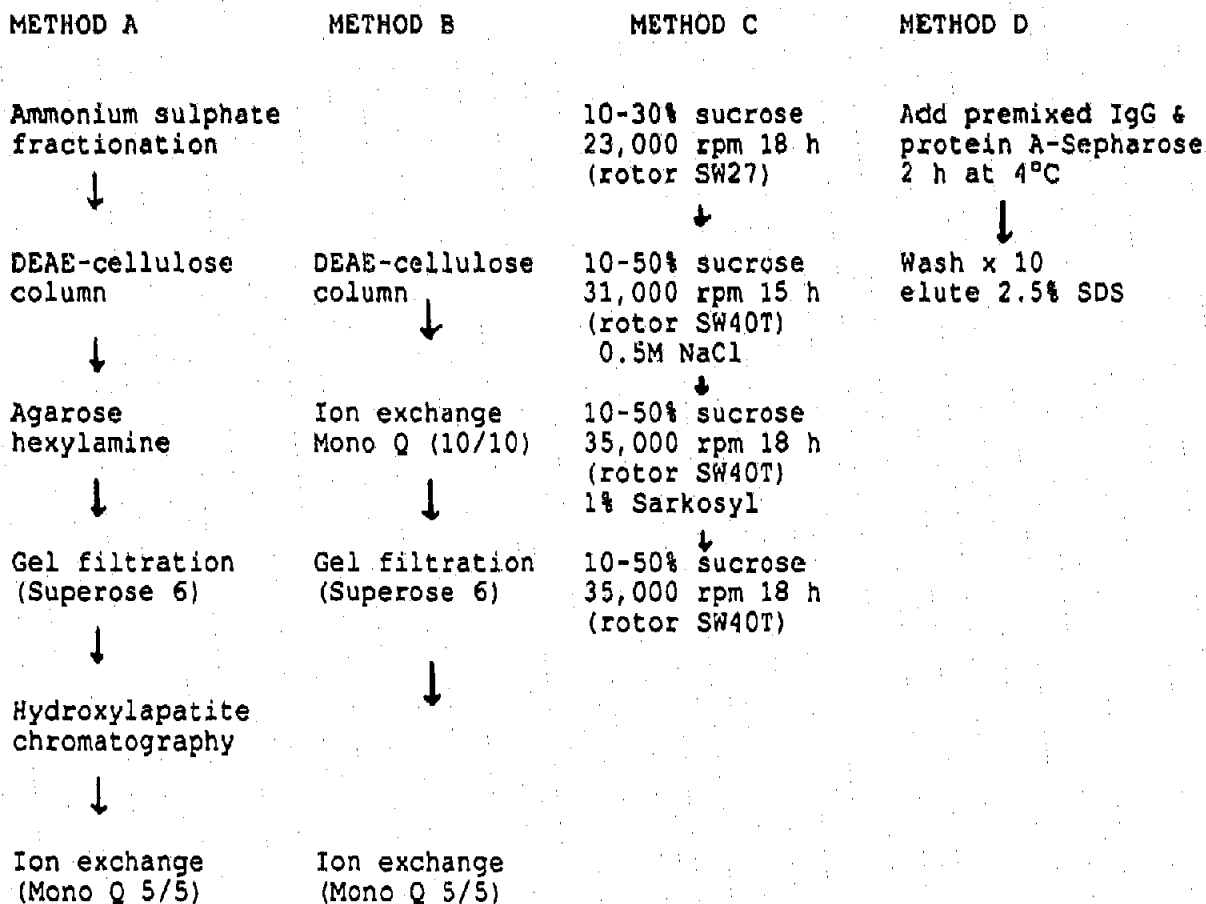
2. MATERIALS AND METHODS

MCP was purified from fresh or frozen rat livers by the procedures outlined in Fig. 1 (methods A, B, C and D). Purification procedures were carried out at 4°C except for FPLC which was at room temperature. In method A, MCP was purified by a conventional chromatographic procedure as described by Rivett [1]. The ammonium sulphate fractionation and hydroxylapatite chromatography have been suspected previously of damaging the RNA content of this particle [16]. Therefore in method B these steps and the agarose-hexylamine chromatography were omitted and an additional Mono Q ion exchange step was included. In method C, MCP was purified by consecutive sucrose gradients in a similar manner to that employed by Schmid et al. [3] with modifications: the buffer used was 20 mM Hepes/KOH pH 8, 10 mM MgCl₂, 10 mM KCl, 0.1% diethyl pyrocarbonate and in some cases a fourth sucrose gradient centrifugation was performed. In method D, MCP was purified from a 100 000 × g supernatant preparation by immunoprecipitation, as described by Hendil [17]; polyclonal IgG preparations against rat liver MCP were as described [18]. Protein concentrations (methods A, B and C) were estimated by comparison with bovine serum albumin as standard using the BioRad protein assay reagent [19], and their purity checked by polyacrylamide gel electrophoresis (PAGE).

Putative RNA was extracted from MCP preparations by the addi-

Figure 1: Purification of the multicatalytic proteinase (MCP) from rat

liver.

METHOD A	METHOD B	METHOD C	METHOD D
Ammonium sulphate fractionation		10-30% sucrose 23,000 rpm 18 h (rotor SW27)	Add premixed IgG & protein A-Sepharose 2 h at 4°C
↓		↓	↓
DEAE-cellulose column	DEAE-cellulose column	10-50% sucrose 31,000 rpm 15 h (rotor SW40T) 0.5M NaCl	Wash x 10 elute 2.5% SDS
↓	↓	↓	
Agarose hexylamine	Ion exchange Mono Q (10/10)	10-50% sucrose 35,000 rpm 18 h (rotor SW40T) 1% Sarkosyl	
↓	↓	↓	
Gel filtration (Superose 6)	Gel filtration (Superose 6)	10-50% sucrose 35,000 rpm 18 h (rotor SW40T)	
↓	↓		
Hydroxylapatite chromatography			
↓			
Ion exchange (Mono Q 5/5)	Ion exchange (Mono Q 5/5)		

tion of proteinase K (1 mg/ml) and SDS (0.1%), and incubation at 37°C for 1 h, followed by the addition of phenol/chloroform/iso-amyl alcohol at 60°C and ethanol precipitation [21]. 3' end labeling was performed on extracted RNA with ^{32}P-labeled pCp in a reaction catalysed by T4 RNA ligase [22].

3. RESULTS AND DISCUSSION

MCP preparations A, B and C appeared as single bands on native protein gels (an example is shown in Fig. 2A). MCP purified by method D could not be included in analyses of the native enzyme because 2.5% SDS was added during the preparation to release the IgG-MCP complex from protein A-Sepharose. Specific activities for preparations A, B and C were measured with two different substrates (Table I). The values obtained were similar for each preparation and within the range expected. All four preparations produced a set of 8-10 protein bands on SDS-PAGE within the 21 000 to 35 000 Da range characteristic of MCP (Fig. 2B). Higher molecular mass protein bands (65 000 to 110 000 Da) were observed in some of the preparations

with all purification protocols. Similar bands to those detected here have been reported by others [20]. Whether they are contaminants or an integral part of the MCP has yet to be determined. Samples prepared by 3 sucrose gradient centrifugations (lane d) contained more high molecular mass polypeptides than those preparations purified by 4 sucrose gradient centrifugations (lane e). It was difficult to determine proteinase purity for preparation D due to the presence of IgG (lane f). The UV spectra for the 3 preparations were similar with a peak at 278 nm (data not shown). The ratio of absorbance at 280 nm to that at 260 nm was 1.8, 2.0 and 1.5 for A, B and C, respectively. It was concluded from previously reported $A_{280}:A_{260}$ values of 1.94 and 1.6 that the RNA content of MCP must be less than 0.1% of particle mass [14,15].

RNA extracted from samples of the different proteinase preparations were run on denaturing and native polyacrylamide gels (Fig. 3). Several bands of RNA were seen when MCP was prepared by immunoprecipitation (Fig. 3A, lane d). This pattern is similar to total

A

B

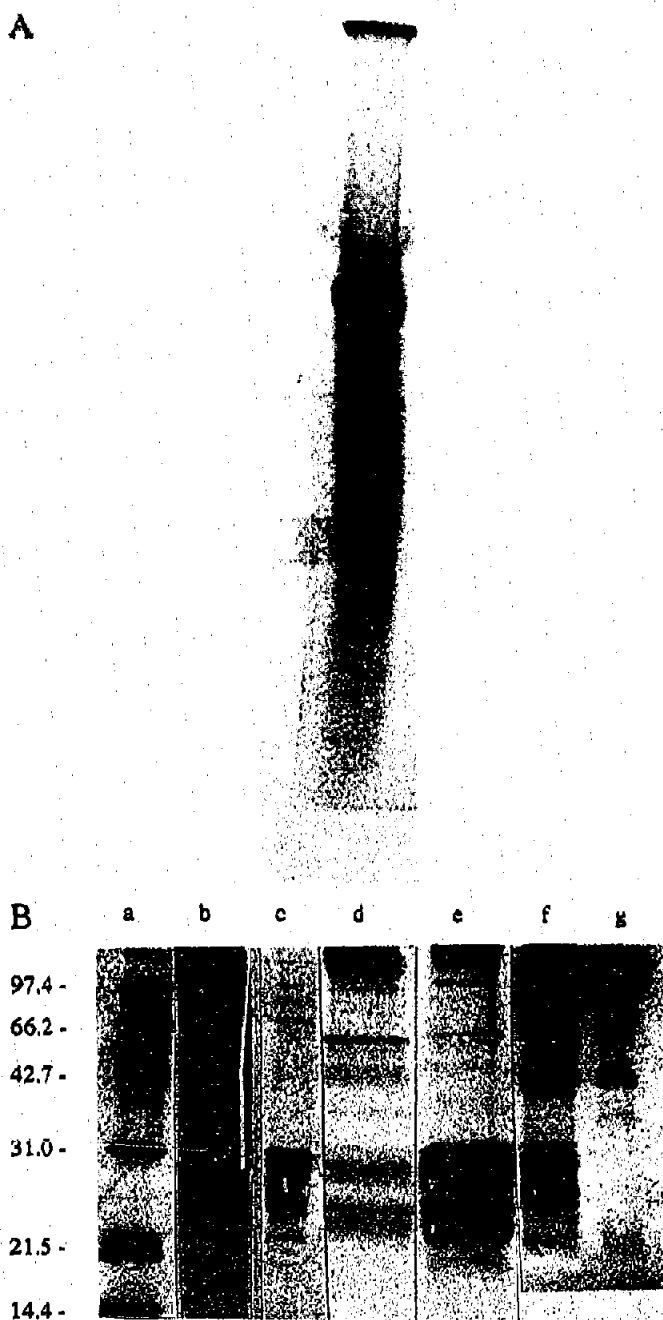|	a	b	c	d	e	f	g
97.4 -							
66.2 -							
42.7 -							
31.0 -							
21.5 -							
14.4 -							

Fig. 2. Polyacrylamide gel electrophoresis of the protein constituents of multicatalytic proteinase preparations. (A) Non-denaturing 5% polyacrylamide gel as described by Davis [27]. MCP purified by method B and stained with Coomassie blue. (B) Electrophoresis of MCP on SDS 15% polyacrylamide gels with 3% polyacrylamide stacking gel as described by Laemmli [28]. Lane a, molecular mass markers (BioRad); lane b, MCP purified by method A; lane c, MCP purified by method B; lane d, MCP purified by method C3; lane e, MCP purified method C4; lane f, MCP purified by method D; lane g, IgG control. All lanes were silver stained.

HeLa cell RNA (not shown). After the fourth sucrose gradient, the MCP produced by method C showed only one band of RNA (Fig. 3A, lane e). Usually, only one band of RNA was observed for the two chromato-

graphic methods (A and B) (Fig. 3B, lanes a and b), but on one occasion some minor additional bands were detected (Fig. 3C). In this case MCP was prepared by method B, followed by an additional gel purification step. RNA was extracted from protein preparations before and after this additional step and radiolabelled. The RNA bands remained associated with MCP even after this extra purification.

Protein samples were taken from different stages during the purification of MCP by method B in order to investigate the RNA present at each step. RNA was extracted from samples containing the same amount of protein and radiolabeled as above. The results demonstrate a specific enrichment of the 80 nucleotide RNA species by the MCP purification protocol (Fig. 3D).

RNA of this length was detected consistently. On denaturing gels this invariant RNA species migrated at the same rate as a molecule of about 80 nucleotides when compared with the DNA markers (Fig. 3A). On native gels the migration of the band was equivalent to that of a double-stranded DNA marker of 50-60 base pairs (Fig. 3B). Thin layer chromatography was performed as described [23] on RNA bands from preparations A and B in order to determine the 3' end base. For both, adenosine 3'-monophosphate was detected (not shown).

The ability of MCP to bind to RNA non-specifically was investigated with rat liver MCP (method A) and HeLa cell RNA. Radioactively-labeled RNA incubated with a preparation of MCP did not bind to the MCP after 30 min, i.e. immunoprecipitated pure MCP did not contain any radioactivity. These investigations tentatively suggest that MCP does not bind non-specifically to RNA.

The amount of RNA detected in MCP was estimated with a laser densitometer by comparing the amount of RNA observed on an autoradiograph with different concentrations of tRNA, 3' end labeled as above. Assuming that MCP RNA was labeled with similar or lower efficiency than tRNA, then this comparison provides a minimum value for the molar ratio of RNA

Table I

Specific activities of MCP prepared by three different methods

Purification protocol	Activity	
	AAF-AMC (nmol/μg/min)	LSTR-AMC
Method A	3.7	1.3
Method B	5.3	1.6
Method C	2.7	1.6

Assays were carried out with 20 μM substrate: AAF-AMC, Ala-Ala-Phe-7-amido-4-methylcoumarin; LSTR-AMC, N-t-Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin as described previously [26].

molecules to MCP. The ratio of RNA molecules (80 nucleotides) to MCP particles was less than one to one thousand. Visual comparisons of RNA from other preparations indicate that the quantity of RNA detected with method B is similar to that found with methods A and C4. Preparations C3 and D contained far more RNA, and as shown (Fig. 3A) there was more than one species of RNA. These preparations also contained more high molecular mass protein bands on an SDS-PAGE, indicating contamination or co-purification of RNP or RNA. Thus for the 3 different stringent purification protocols far less than one molecule of
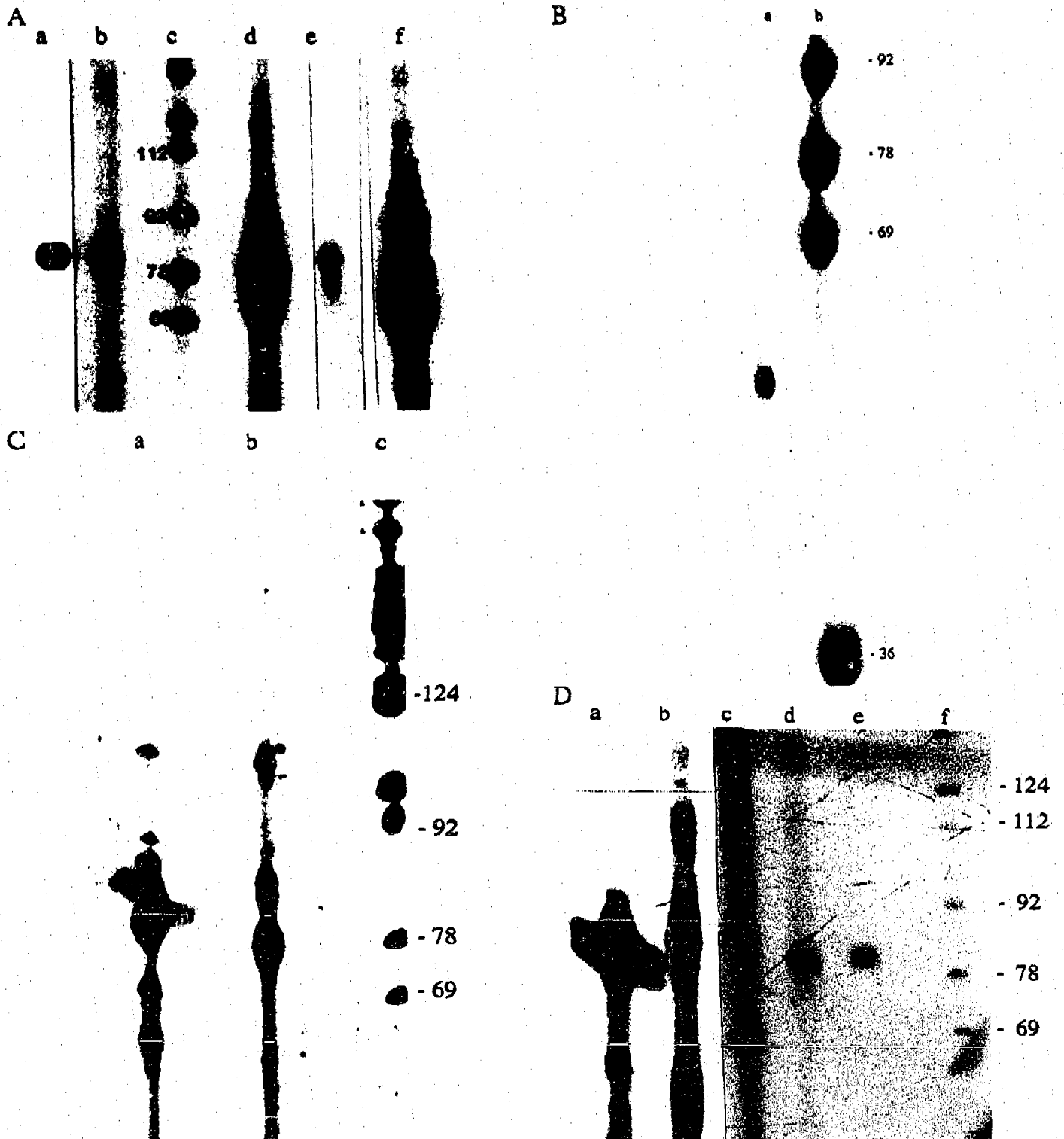
RNA was observed per protein particle. This suggested either that there are at least two populations of rat liver MCP, one containing one species of RNA, 80 nucleotides in length, and the other not, or that much of the RNA is being damaged during protein purification and the RNA detected here only represents a portion of the total RNA. The latter seems unlikely because similar levels of RNA were detected in preparation C (where diethyl pyrocarbonate was included to protect against ribonucleases) and in preparation B where no such precautions were taken. This observation also suggests that MCP-associated RNA is protected from nuclease attack by proteins. This agrees with observations of Divena et al. [24] which suggests that RNA in prosomes is resistant to nuclease attack.

The observation of an 80 nucleotide RNA species associated with rat liver MCP agrees with previous reports on RNA isolated from prosomes and 20 S or 19 S particles purified by consecutive sucrose gradients. At least one species of RNA was detected in the 50–120 nucleotide size range [2,3,9,10,11,16,25], most frequently in the 70–90 nucleotide range. The quantity of RNA detected, however, was generally more than that observed here. From buoyant density estimations in caesium sulphate, protein to RNA mass ratios of 4:1, 8:1 and 8.5:1 were estimated [3,8,10,11,25]. Partial sequences of RNA from such a preparation of *Drosophila* prosomes found one RNA which was 74% identical to human U6 snRNA [8]. The RNA isolated in our work is unlikely to be U6 RNA due to the presence of adenosine at the 3' end. This, together with its length, suggests a tRNA molecule. Several bands of RNA in the 80 and 100 nucleotide range were also observed from rat liver MCP purified by immunoprecipitation or CsSO₄ density gradients [4].

The difficulty now is in deciding which RNA species are intrinsic to MCP and which are artifacts of co-purifications. From the results presented here it is clear that very highly purified preparations of rat liver MCP do contain a species of RNA of 80 nucleotides which cannot be removed by further electrophoretic purification of the particles. The RNA may be bound tightly in only a small proportion of MCP particles. If, as this implies, MCP preparations are not homogeneous, it is possible that a common structural element is adapted in different particles for different functions and that this

non-homogeneity explains the diverse functions attributed to MCP-like particles.

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Fig. 3. Autoradiographs of polyacrylamide gel electrophoresis of RNA extracted from MCP. RNA was extracted and labeled in vitro at its 3' end with [³²P]pCp. (A) 7 M urea, 8 M formamide 10% polyacrylamide gel autoradiographs of RNA extracted from MCP. Lane a, MCP purified by method A; lane b, MCP purified by method B; lane c, HindIII cut lamda DNA markers; lane d, MCP purified by method C3; lane e, MCP purified method C4; lane f, MCP purified by method D. (B) 10% polyacrylamide non-denaturing gel autoradiographs of RNA extracted from MCP. Lane a, MCP purified by method B; lane b, HindIII cut lamda DNA markers. (C) 10% polyacrylamide denaturing gel autoradiographs of RNA extracted from MCP. Lane a, MCP purified by method B; lane b, MCP purified by method B with an additional gel purification step by loading onto a non-denaturing protein gel and eluting in 10 mM triethanolamine/HCl, pH 7.6, 2 mM EDTA and 0.5% SDS; lane c, HindIII cut lamda DNA markers. (D) Autoradiographs of RNA extracted from protein samples taken during different stages of the purification method B. Lane a, pre-DEAE celluloase column; lane b, pre-Mono Q 10/10 chromatography; lane c, pre-gel filtration; lane d, pre-Mono Q 5/5 chromatography; lane e, final preparation; lane f, HindIII cut lamda DNA markers. The photograph of lanes a, b and c was taken of a shorter exposure autoradiograph to the photograph of lanes d, e and f.