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Cross-linked informofers

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ABSTRACT

The proteins of 30S RNP particles containing pre-mRNA (hnRNA) were cross-linked with bifunctional reagents (dimethyl-suberimidate and dimethyl-3,3'-dithiobispropionimidate). Further treatment with 1 or 2 M NaCl dissociates all RNA from protein. However, a significant part of protein particles - informofers being cross-linked survived high salt treatment. Their sedimentation coefficients were close to those of original particles. No RNA could be detected in the informofers even after labeling the cells with a precursor for a long period of time. Sodium dodecylsulfate or urea dissociated cross-linked informofers into oligomeric polypeptides. They could be dissociated by ß-mercaptoethanol treatment if a reversible cross-linking reagent had been used. The resulting polypeptides were represented by informatin. RNP particles (30S RNP or poly-particles) were reconstituted upon mixing of cross-linked informofers with pre-mRNA and removal of 2 M NaCl.

INTRODUCTION

It has been shown previously that nuclear pre-mRNP (hnRNP) particles are represented by chains of repeating units and can be easily cleaved by mild RNAase treatment into monomers, or 30S particles /1,2/.

Several data indicate that each 30S particles consists of protein core (referred as "informofer") and a chain of RNA about 600 nucleotides in length wrapped on the surface of an informofer /1-4/.

30S particles can be dissociated into RNA and protein by 1 or 2 M NaCl treatment /5,6/. The liberated protein is found in two states: either as informofers, i.e. particles comparable in size with non-dissociated 30S RNPs, or as a low molecular weight material which corresponds to polypeptides forming RNP

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/6/. These polypeptides which are called "informatin" have a molecular weight of about 40,000. They are rather homogeneous and give rise to one component in urea and two or three close bands in SDS gel electrophoresis /5-7/. The informofers and informatin are present at equilibrium in 2 M NaCl and their relative amount is a function of protein concentration. The reassociation of any of them with RNA give rise to 30S RNP particles formation. However, only with non-dissociated informofers the real reconstitution take place and structure of RNP particles formed seems do not differ significantly from original one /6/.

The results of these experiments are interpreted as proof of RNA localization on the surface of informofers. However, the reversible dissociation of informofers in the course of reconstitution procedure cannot be excluded. Therefore in the present work we treated the proteins of 30S particles with the bifunctional reagents dimethylsuberimidate (DMSI) or dimethyl-3,3'-dithiobispropion imidate (DMDTPI) to obtain cross-linked informofers unable to dissociate. This has been achieved and we have found that RNA can be easily removed from RNP without dissociation of an informofer as well as put back to a cross-linked informofer with reconstitution of the original RNP. These data give further proof for the pre-mRNP structure postulated previously /2-4/.

MATERIAL AND METHODS

Material. 30S pre-mRNP particles were obtained from rat liver or mouse Ehrlich ascites carcinoma cells. The isolation of cell nuclei and pre-mRNA containing RNP particles from them has been performed essentially as was described previously /2,8,9/. Rat liver cells were labeled by the intraperitoneal injection of ^{32}P orthophosphate (1-2 mCi per animal) or ^{3}H - uridine (100-200 μCi per animal). The labeling of RNP particles from mouse Ehrlich ascites carcinoma cells was performed by their incubation in vitro with a radioactive precursor in the Eagle solution. For labeling the protein, the mice were injected intraperitoneally with ^{3}H protein hydrolysate (Checho-

slovakia) on the 5-th and 6-th days after transplantation of ascites. (The total dose was equal to 500 μCi per mouse).

Pre-mRNA for reconstitution experiments was isolated either from 30S RNP by hot phenol detergent extraction /2,10/ or from intact cells by hot phenol fractionation method /8,11/.

Cross-linking procedure is essential as was outlined in refs. 12 and 13. The 30S sucrose gradient peak was collected and after short dialysis against STM* buffer (100 mM NaCl, 10 mM TEA-HCl, pH 7.0, 1 mM $MgCl_2$) was treated with DMSI or DMDTPI at pH 7.8. The reaction was performed at 4°C for 4 to 12 hrs. The ratio of cross-linking reagent to protein was 10:1.

Isolation of cross-linked informofers. The reaction mixture (usually 1-2 ml) was put directly onto a 15-30% sucrose gradient containing STM buffer and 1 or 2 M NaCl, and ultracentrifuged at 25,000 rpm in a SW-27 rotor for 15-17 hrs. The fractions were collected and analysed for optical density at 260 nm and 230 nm and also for radioactivity. The rapidly sedimenting material (≈30S) was collected from the gradient and used in further experiments.

Reconstitution experiments. Cross-linked informofers obtained from a sucrose gradient containing high concentration of NaCl were mixed either with ^3H labeled pre-mRNA isolated from Ehrlich ascites carcinoma cells by the hot phenol fractionation procedure or with RNA isolated from labeled nuclear 30S RNP particles, dialyzed overnight against STM buffer, and ultracentrifuged in a 15-30% sucrose gradient in the same buffer. The fractions were collected and analysed as described above.

Ultracentrifugation in performed CsCl density gradient was performed either with the original 30S particles or with reconstituted particles fixed by 4% CH_2O /5/, or with unfixed cross-linked particles. The material was ultracentrifuged in a SW-40 rotor of Spinco ultracentrifuge at 35,000 rpm for 20 hrs at 16°C.

Polyacrylamide gel electrophoresis of nuclear RNP proteins was performed according to the method of Bhorjee and Pederson

/15/. For radioactivity determination, the gels were cut into 2 mm slices which were dissolved in a NCS solubilizer and counted in a dioxane scintillator with the aid of an Intertechnique SL-30 model.

RESULTS

In the first series of experiments, 30S particles isolated from rat liver were treated with increasing concentration of DMSI for different periods of time. If the concentration of 30S RNP particles was less than 0.1 mg/ml, the optimal condition for DMSI treatment were the following: 0.1% DMSI for 10 hrs at 4°C. In this case, the higher cross-linking was obtained and no loss of particles due aggregation of them took place. After recentrifugation in a sucrose gradient, almost all of the material was recovered in the 30S peak (Fig.1c) while with untreated particles, some of the material was shifted to the light zone upon recentrifugation (Fig. 1b). Then concentrated NaCl was added to treated and untreated particles to a final concentration of 2 M and the mixture was ultracentrifuged in a sucrose gradient containing 2 M NaCl. In the case of rather low protein concentration ($\leqslant$0.1 mg/ml), practically the whole protein of untreated particles was recovered in the upper region of the gradient (~3-4S) (Fig.1d), in agreement with the previous results /6,14/. However, after preincubation with a cross-linking reagents a significant part of the particles became resistant to a high salt solution and sedimented in the 30S region (Fig. 1e). It is noteworthy that RNA was completely removed from protein in these conditions as followed from the shift of OD_{260} to the light region (~3-6S).

The same results were obtained with 30S particles isolated from Ehrlich ascite cell nuclei labeled in vitro with a mixture of ^{3}H aminoacids (see Methods) and with ^{32}P orthophosphate (Fig. 2). Again during resedimentation in 2 M NaCl of cross-linked particles a significant part of the protein label remained in the 30S zone whereas almost all RNA dissociated from the particles and moved to the light zone of the gradient (Fig. 2b). Without cross-linking of the protein core

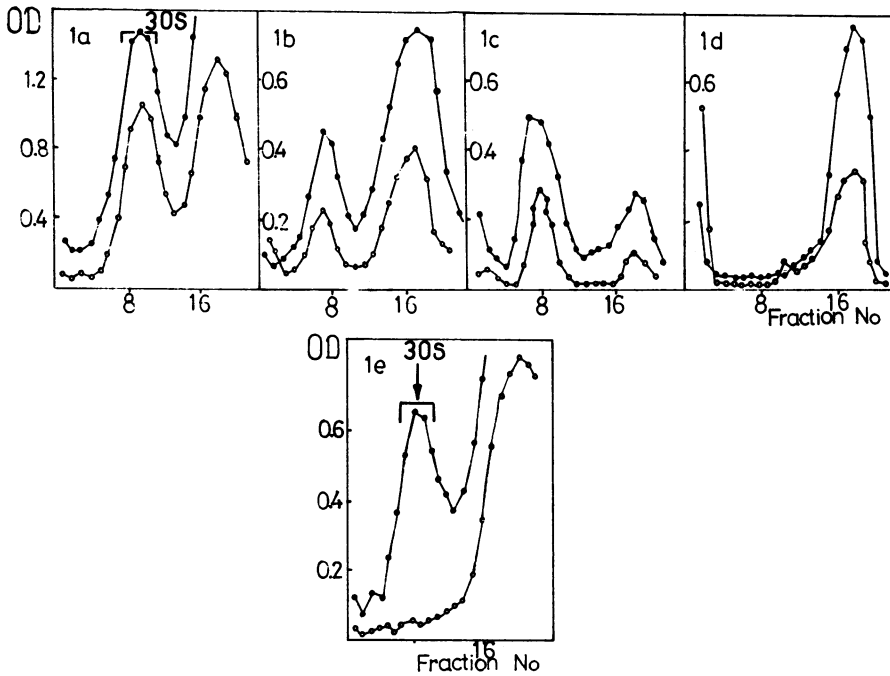

Fig. 1. Resedimentation of rat liver 30S RNP particles before and after cross-linking with DMSI.

Cross-linking was performed for 10 hrs at 4°C; the protein and DMSI concentrations are 100 µg/ml and 1 mg/ml, respectively.

a) Sedimentation of nuclear extract in a 15-30% sucrose gradient.

b) Resedimentation of untreated 30S particles under the same conditions.

c) Resedimentation of 30S RNP particles cross-linked with DMSI in a sucrose gradient containing STM buffer.

d) Resedimentation of isolated 30S RNP particles in a sucrose gradient containing 2 M NaCl.

e) Resedimentation of cross-linked 30S RNP particles in a sucrose gradient containing 2 M NaCl (the zone for further experiments is marked).

o-----o - OD_{260}

•——• - OD_{230}

the whole radioactive proteins were also detected in this zone. Dissociation of RNA from cross-linked particles in high salt condition was confirmed further by decreasing the buoyant

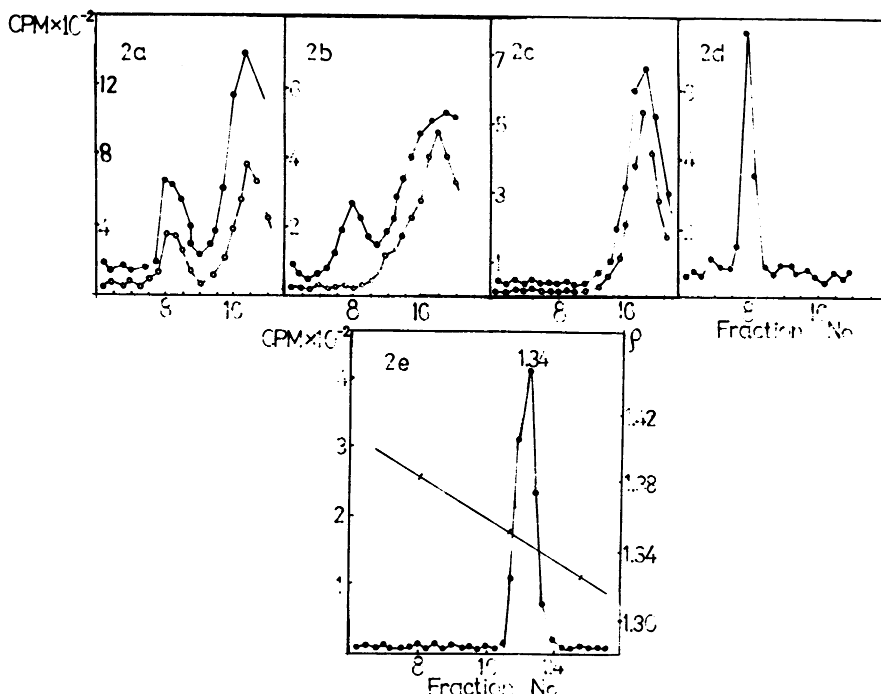

Fig. 2. Sedimentation of mouse carcinoma 30S particles containing labeled RNA (^{32}P) and protein (^{3}H) before and after DMSI treatment.

a) Ultracentrifugation of the double-labeled nuclear extract in a 15–30% sucrose gradient in STM buffer.

b) Recentrifugation of 30S particles isolated from (a) and cross-linked with DMSI in a sucrose gradient containing 1 M NaCl.

c) The same as (b) but 30S RNP particles were not cross--linked.

d) Recentrifugation of the 30S zone isolated from (b) in a sucrose gradient containing STM buffer.

e) Centrifugation of the material of cross-linked 30S particles isolated from (b) in a CsCl density gradient.

—•—•— ^{3}H-protein

—o—o— ^{32}P-RNA

density of this material from 1.40 to 1.34 (Fig. 2e). Thus, cross-linking of protein with bifunctional reagents stabilizes the informofer but does not interfere with the dissociation of

RNA from it.

On the basis of distribution of in vivo labeled protein between the 30S and 3S zones, one may conclude that from 30 to 70% of informofers became resistant to dissociation under our conditions of DMSI treatment (see Fig. 1 and 2).

It is important that 30S protein particles are not at equilibrium with dissociated material. Actually, if they are collected and recentrifuged in a sucrose gradient, the whole material is concentrated in the 30S region. No additional dissociation takes place (Fig. 2d). On the other hand, treatment of particles with either 0.5% SDS or 4 M urea shifted the protein into the light peak which is relatively heterogeneous in these conditions (data not shown).

The dissociated protein has been analysed with the aid of SDS gel electrophoresis. After cross-linking, one can observe oligomers of informatin (Fig. 3b). Oligomer patterns depend on the conditions of the cross-linking reaction. Usually most of the material is represented by dimers and trimers of informatin. In several cases, one can see pentamers and even heptamers, but it is not necessary to obtain high oligomers of informatin for complete stabilization of informofers. Formation of dimers and trimers is enough for complete stabilization of a free informofer in a salt solution. This possibly depends on the cooperative character of informofer dissociation. Rather few cross-links stabilize the whole structure in the absence of strong denaturing agents.

The question arises whether particles which become cross--linked represent the total population or some fractionation takes place in the course of the reaction with a bifunctional reagent. To answer this question, cross-linking was conducted with the aid of the reversibly acting reagent DMDTPI. The NaCl--sensitive material and the NaCl-resistant material were collected after sucrose gradient ultracentrifugation in 2 M NaCl, treated with mercaptoethanol and analysed by polyacrylamide gel electrophoresis. Exactly the same electrophoretic patterns were obtained in both cases. Two main bands (~40,000 daltons) of informatin were observed and unsignificant quantity of heavy components corresponding mainly to dimers in the case

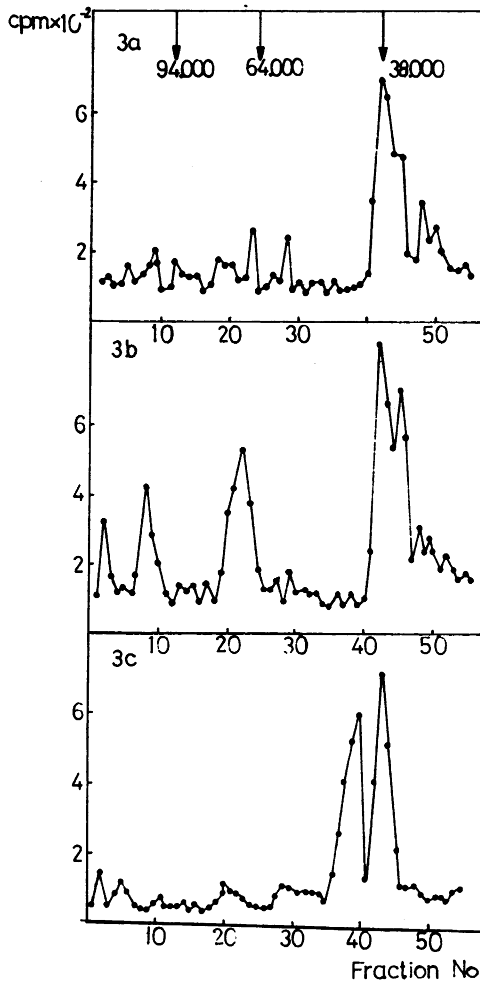

Fig. 3. SDS polyacrylamide gel electrophoresis of proteins from cross-linked and untreated 30S RNP particles.

The informofers labeled by ^{3}H-amino acids and cross--linked with DTDMPI were treated with SDS, mixed with an excess of unlabeled protein from untreated RNP and electrophoresed in an SDS gel.

Another sample was incubated with mercaptoethanol before electrophoresis. The gels were analyzed as described in Methods.

a) Distribution of the carrier protein (informatin) stained with Coomassie.

b) Distribution of the label in the protein from cross--linked particles.

c) The same but after mercaptoethanol treatment.

of material collected from 30S zone (Fig. 3c).

Thus, the cross-linking reaction does not fractionate particles according to their protein composition.

The absence of internal RNA from cross-linked informofers.

First, it was postulated that informofers represented compact protein particles to whose surface pre-mRNA was bound /1,2,4/. Later it was found by Sekeris et al. /16,17/ and confirmed by one of us (V.Prosvirnin, unpublished/ that long labeled RNA of the 30S particles was more resistant to RNAase than the rapidly labeled one. One explanation is that a special low molecular weight metabolically stable RNA is localized within the informofer and therefore it is more resistant to RNAase than pre-mRNA /16,17/. Another possible explanation is that metabolically stable RNA accumulated in the course of processing is enriched in the double-stranded region and this makes it more resistant to RNAase treatment. The existence of dsRNA in 30S RNP has already been demonstrated /18,19/.

Cross-linked informofers seem to be a good system for checking the existence of RNA within the informofer. As they do not dissociate during isolation from 30S particles, one may expect that internal RNA resides within the particles.

30S particles were prepared from rat livers labeled with ^{32}P orthophosphate for 16 hrs and with ^{3}H uridine for 60 min, and informofers were obtained in 1 M NaCl after cross-linking with DMSI.

As one can see from Fig.4b, in 1 M NaCl, almost no radioactive RNA is presented in the 30S peak. Some label found depends possibly on the presence of free aggregated RNA in the 30S region. The 30S material was collected, fixed additionally with 2% CH_2O, and ultracentrifuged in a CsCl density gradient. The protein was banded at a density of 1.34 g/cm^3 (not shown) characteristic for informofers obtained without cross-linking /6/. Neither rapidly labeled nor metabolically stable RNA was found in this band. Thus, cross-linked informofers do not contain RNA and this indicates that there is no internal structural RNA in 30S pre-mRNP particles.

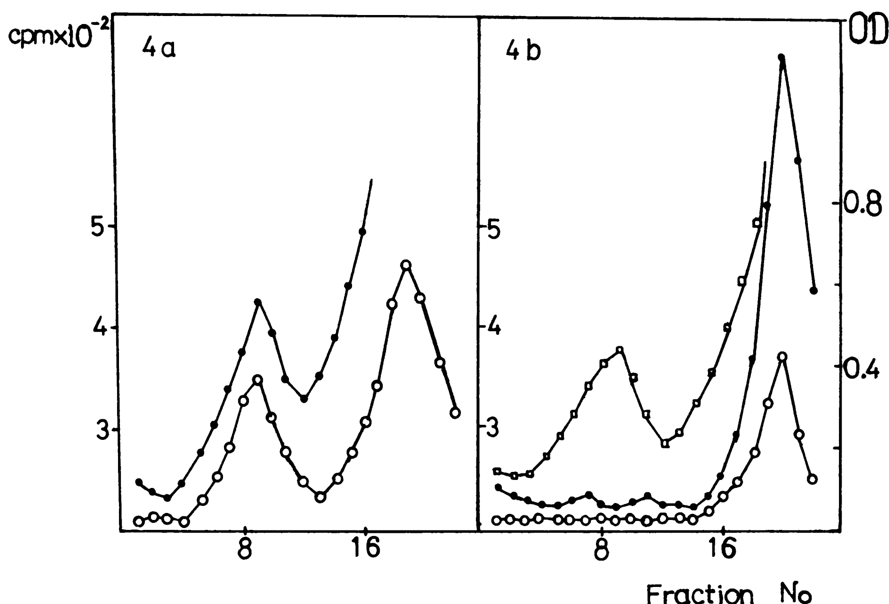

Fig. 4. Sedimentation of cross-linked 30S particles containing short pulse and long labeled RNA.

Rats were injected with ^{32}P-orthophosphate 16 hrs before killing and 60 min pulse of ^{3}H-uridine. 30S particles isolated from the nuclear extract were cross-linked with DMSI and recentrifuged in sucrose gradient containing 1 M NaCl.

a) Sedimentation of nuclear extract in sucrose gradient containing STM buffer.

b) Sedimentation of cross-linked 30S particles isolated from (a) in sucrose gradient containing 1 M NaCl.

-o-o-o- A_{230}
—•—•— ^{3}H
—o—•— ^{32}P

Reconstitution experiments

Finally, reconstitution experiments were performed with cross-linked informofers. These were mixed with labeled RNA, and 2 M NaCl was removed by dialysis against a low ionic strength buffer.

In the first experiments, RNA isolated from 30S RNP par-

ticles which were obtained from Ehrlich ascite cells labeled with ^{3}H uridine was used. As one can see from Fig. 5a, a significant proportion of labeled RNA shifted to the 30S zone of the sucrose gradient and sedimented together with cross-linked informofers. The same experiments were repeated with ^{3}H pre--mRNA isolated by the hot phenol fractionation method from ascite cells. In this case, the size of RNA used in the reconstitution reaction was higher (15-18S) than that of RNA isolated from 30S RNP particles. After reconstitution, the distribution of RNA in the sucrose gradient differed from the above mentioned. Two discrete peaks are seen on Fig. 5b which probably correspond to mono and dimeric RNP particles. This assumption was confirmed by examination of the labeled material collected from this peak in a CsCl density gradient after

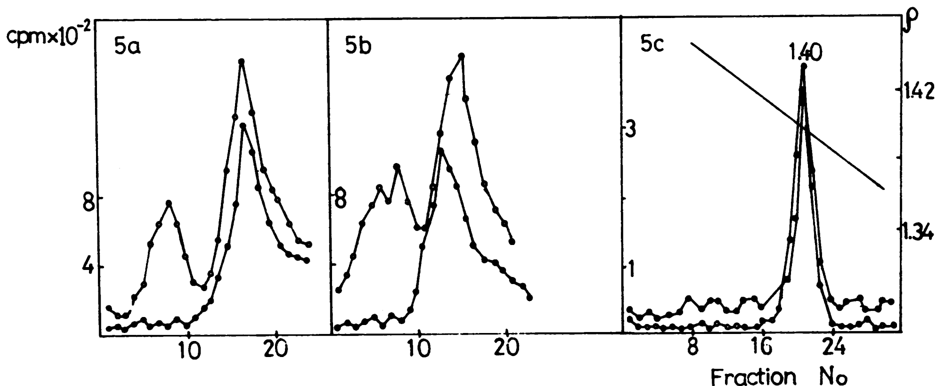

Fig. 5. Reconstitution of RNP particles from cross--linked informofers and pre-mRNA.

a) Sucrose gradient in STM of RNP particles reconstituted from cross-linked informofers (Fig. 1e) with RNA isolated from 30S RNP particles labeled with ^{3}H-uridine.

b) The same as (a) but ^{3}H-pre-mRNA used for reconstitution was isolated from Ehrlich ascites cells by the hot phenol fractionation method.

c) The CsCl density gradient of RNP particles reconstituted from cross-linked informofers and RNA.

●——● Sedimentation of ^{3}H-RNA taken for reconstitution and centrifuged in a parallel tube.

-o-o- The ^{3}H-label of the material obtained after reconstitution.

formaldehyde fixation. In all of the cases, the density was the same and equal to that of the original 30S RNP particles (1.40 g/cm^3) (Fig. 5c).

Also, like the original particles, the reconstituted material completely dissociated into RNA and protein if formaldehyde fixation had been omitted (data not shown).

DISCUSSION

We found that cross-linked informofers resistant to dissociation into informatin molecules can be easily separated from RNA. 1 or 2 M NaCl removes tha latter completely from the particles. Thus, the compact structure of the particles does not create any topological obstacles to RNA removal and this result strongly supports the surface localization of RNA suggested from the previous results /6,20/.

Furthermore, cross-linked RNA-free informofers can be used for the reconstitution reaction. They efficiently interact with RNA forming monomeric and oligomeric RNP particles similar in their properties to the original RNP. This result again confirms the above statement.

It is very probable that the informofer stabilization effect of the iodination procedure as well as of aging noticed previously /20/ depends on the formation of a limited number of cross-links between informatin molecules in informofers. These may be, for example, S-S bounds which are known to appear readily in informatin solutions. Due to the cooperative character of informofer dissociation these cross-links may prevent the latter process completely.

The availability of cross-linked informofers free of RNA and able to interact with RNA opens new lines for attacking the problem of the structure and function of nuclear pre-mRNA particles.

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