EXCESS OF MICROCOCCAL NUCLEASE MAY HARM THE EXOGENOUS mRNA IN THE mRNA-DEPENDENT RABBIT RETICULOCYTE CELL-FREE SYSTEM

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1. Introduction

The mRNA-dependent rabbit reticulocyte cell-free system [1] has been found useful for the study of in vitro translation of several mRNAs ([1–3], Van Tol and L.v.V.-D., submitted). We chose this system to produce μg amounts of the gene products of the tripartite genome of alfalfa mosaic virus (AMV) [4]. One of the problems encountered when scaling up the in vitro protein synthesis was the large amount of relatively expensive micrococcal nuclease required according to the procedures in [1–3].

We report here that on a weight basis the concentration of micrococcal nuclease can be substantially reduced without affecting the proper inactivation of endogenous mRNA. Moreover, we show that the use of high concentrations of micrococcal nuclease may cause the production of shorter peptide chains.

2. Materials and methods

2.1. *Preparation of mRNAs*

RNA was obtained from the purified nucleoprotein components as in [5,6].

2.2. *Preparation of the mRNA-dependent rabbit reticulocyte lysate*

Rabbits were made anaemic using *N*-acetylphenylhydrazine and the reticulocyte lysate was prepared and fractionated as in [7]. Fractions were stored at −80°C. Before use each fraction (0.4 ml) was pretreated for 10 min at 20°C with: 6 μl 1 mM hemin in 90% ethylene glycol, containing 50 mM Tris–HCl (pH 8.0); 4 μl 5 mg/ml creatine kinase; 10 μl 0.5 M creatine phosphate; 30 μl 0.5 mM each of the 19 unlabeled amino acids; 5 μl 100 mM $CaCl_2$; micrococcal nuclease (EC 3.1.4.7, Boehringer) as indicated in the figure legends in 35 μl H_2O. The activity of the micrococcal nuclease was inhibited by the addition at 0°C of 10 μl 100 mM of ethylene-glycol-bis(2-aminoethyl ether)*N,N'*-tetraacetic acid (EGTA).

2.3. *In vitro protein synthesis*

Incubation mixtures (35 μl) contained: 68% (v/v) pretreated lysate; 70 mM KCl; 0.2 mM spermidine; from 0.2–0.5 μCi L-[^{35}S]methionine; 25 μg/ml wheat germ tRNAs [7] and 10 μg of the indicated mRNA. Incubation was for 2 h at 30°C.

Half of the sample was used for determination of total incorporation by trichloroacetic acid precipitation, after decoloration of the sample with 1.5 ml 0.17 M H_2O_2 in 0.35 M NaOH for 5 min at 90°C. The precipitate was collected by filtration of Whatman GF/C glass filter, washed and counted. Half of the sample was used for the analysis of the nature of the translation products by electrophoresis on 11% or 15% polyacrylamide–0.1% SDS slab gels together with protein markers as in [8], followed by fluorography [9].

3. Results and discussion

3.1. *Concentration curve of micrococcal nuclease*

Figure 1 shows the effect of pretreatment of the rabbit reticulocyte lysate with different concentrations of micrococcal nuclease on the endogenous

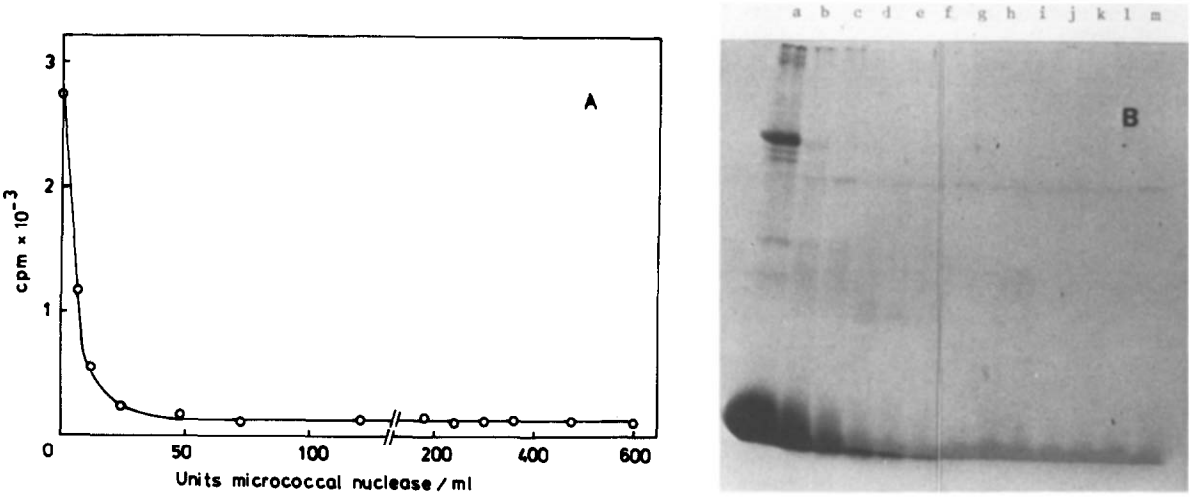

Fig.1. Effect of pretreatment of a reticulocyte lysate with increasing concentrations of micrococcal nuclease on the endogenous protein synthesis. (A) Amino acid incorporation; (B) Product analysis: (a) 0; (b) 6; (c) 12; (d) 24; (e) 48; (f) 72; (g) 120; (h) 180; (i) 240; (j) 300; (k) 360; (l) 480; and (m) 600 units micrococcal nuclease/ml.

protein synthesis. It is evident that pretreatment of the lysate with 50–75 units/ml is sufficient to make the protein synthesis dependent on the addition of mRNA. The same result was obtained with several batches of lysates and two batches of nuclease.

In the literature concerning the mRNA-dependent rabbit reticulocyte system the use of a final concentration of 10 [1] or 70 [2] μg enzyme/ml was reported.

However, the units enzyme/mg protein were not given. Since our batches contained 15 000 and 30 000 units enzyme/mg, 60 units enzyme/ml corresponds to 2 or 4 μg protein/ml.

3.2. *Effect of the concentration of micrococcal nuclease on the in vitro translation products*

All four AMV-RNAs were translated in lysates pretreated with either 60 units or 600 units micrococcal nuclease/ml. These pretreatments did not influence the amount of methionine incorporation. The patterns of the products directed by AMV-RNA 1, 2 and 4 in the two mixtures were identical (fig.2). However, comparison of the products directed by AMV-RNA 3 showed that the 33 000 protein, which was always formed in lysates pretreated with 600 units nuclease/ml, (Van Tol and L.v.V.-D., submitted) was not formed in lysates pretreated with a low amount of nuclease. To see if this difference was due indeed to the nuclease, AMV-RNA 3 was translated in lysates pretreated with increasing amounts of nuclease. From fig.3A it is evident that increasing the concentration of micrococcal nuclease results in the formation of increasing amounts of faster migrating products, especially of a 33 000 protein.

The difference in the products can be due either to degradation of the mRNA by nucleases or to degradation of the product by proteases eventually present in the nuclease preparation. To investigate the latter possibility AMV-RNA 3 was translated in a lysate pretreated with 30 units/ml micrococcal nuclase and the product was incubated with increasing amounts of nuclease. Since this treatment had no effect on the size of products (fig.3B) it seems unlikely that a proteolytic activity is responsible for the appearance of the faster migrating products. We do not know whether the apparent nucleolytic activity in the presence of EGTA is due to traces of Ca^{2+}-independent nucleases present in the enzyme preparation or to a low level of activity of the micrococcal nuclease itself.

The fact that the translation products were influenced by the concentration of micrococcal

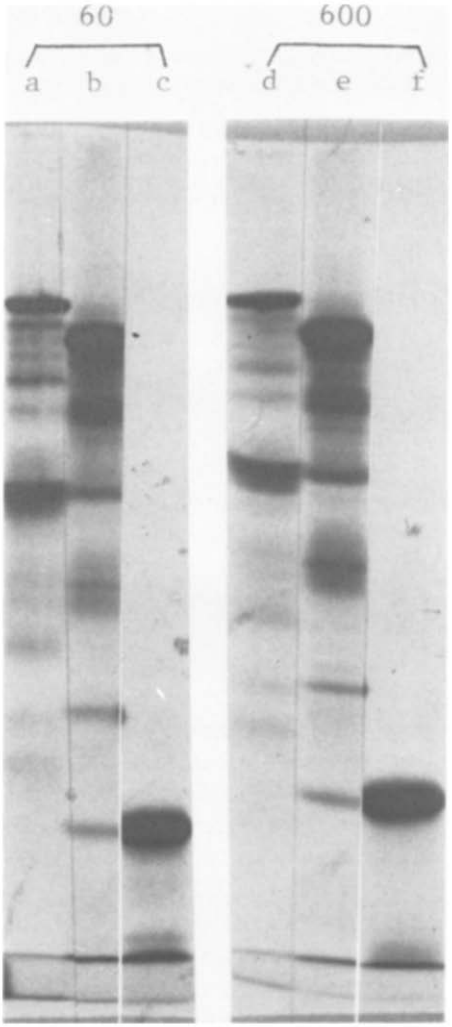

Fig.2. Translation products directed by AMV-RNAs in lysates pretreated with 60 (a, b, c) or with 600 units micrococcal nuclease/ml (e, f, g). (a, d) Products directed by AMV-RNA 1; (b, e) products directed by AMV-RNA 2; (c, f) products directed by AMV-RNA 4. Lanes (a, b, c) and (d, e, f) are from separate slab gels.

nuclease only in the case of AMV-RNA 3 suggests that AMV-RNA 3 contains near the end of the 35 000 cistron a site which is very sensitive for this particular nuclease.

4. Conclusion

Treatment of a rabbit reticulocyte lysate with

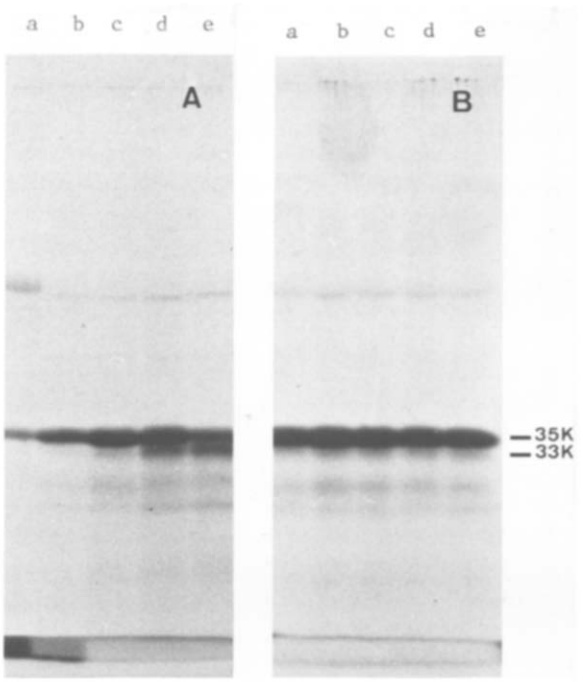

Fig.3. Effect of increasing concentrations of micrococcal nuclease on the products directed by AMV-RNA 3. (A) Effect of pretreatment of the lysate with: (a) 0; (b) 24; (c) 96; (d) 200; and (e) 600 units micrococcal nuclease/ml. (B) Effect of treatment of the translation products directed by AMV-RNA 3 with a final concentration: (a) 20; (b) 44; (c) 106; (d) 190; and (e) 420 units nuclease/ml.

50–75 units micrococcal nuclease/ml is sufficient to make the protein synthesis dependent on addition of exogenous mRNA. Use of higher concentrations of nuclease should be avoided, since this may lead to degradation of the exogenous mRNA.

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