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Detection of a proteolytic activity in the micrococcal nuclease used for preparation of messenger-dependent reticulocyte lysates

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Some, but not all, commercial preparations of micrococcal nuclease used to remove endogenous mRNA from reticulocyte lysates were found to contain proteolytic activity. The protease(s) caused a time-dependent cleavage of the polyprotein primary translation products of genome RNA of several plant viruses, but did not affect the translation products of some other virus mRNA. The activity resulted in the production of smaller proteins and was inhibited by zinc ions. Thus the protease(s) mimicked virus-coded proteases and represents a potential artifact in studies of translation products of virus RNA.

Tomato black ring virus; Translation, in vitro; Proteolytic processing; Micrococcal nuclease

1. INTRODUCTION

One of the expression strategies of positive stranded viral RNAs is in the translation of genome RNA to yield a large polyprotein which is subsequently cleaved by a virus-coded protease to give functional products [1]. Rabbit reticulocyte lysates have been used in many studies of in vitro translation of mRNAs and in the analysis of the pathway of the proteolytic processing of the primary translation products. Pelham and Jackson [2] showed that treatment of reticulocyte lysates by micrococcal nuclease destroys the endogenous RNA and most workers have used such treated reticulocyte lysates.

Each RNA of tomato black ring virus (TBRV), a nepovirus, is a monocistronic RNA and the translation products must be cleaved to give rise to structural and non-structural proteins [3,4]. In our translation studies we have used nuclease-treated lysates, but surprisingly the patterns of the proteins synthesized after longer incubation periods in treated reticulocyte lysates differed with the source of micrococcal nuclease used in the treatment.

This paper reports results that show that some preparations of nuclease contain significant protease activity.

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2. MATERIALS AND METHODS

2.1. *Virus and RNA*

The TBRV is an isolate from Lanarkshire and belongs to the Scottish serotype. RNA was extracted as described [5]. GCMV is the Hungarian grapevine chrome mosaic virus [6]. GCMV RNA was provided by T. Candresse (INRA, Pont de la Maye, France).

RNAs of TMV (tobacco mosaic virus), TYMV (turnip yellow mosaic virus) and AlMV (alfalfa mosaic virus) were a generous gift from M. Pinck (IBMP, Strasbourg). RNAs from BNYVV (beet necrotic yellow vein virus) F13 isolate [7] were provided by K. Richards (IBMP).

2.2. *Micrococcal nuclease*

Sources of micrococcal nuclease were from Boehringer (B), Sigma (S) and Worthington (W). Several batches of S and W nuclease were tested.

2.3. *In vitro protein synthesis conditions*

Rabbit reticulocyte lysates were purchased from Green Hectares (Oregon, Wisconsin) and made RNA dependent using the

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calcium chloride micrococcal nuclease treatment [2]. The lysates supplemented with 25 μM hemin were incubated at 20°C after addition of 10 μg/ml micrococcal nuclease and 1.25 mM $CaCl_2$. After 15 min, the activity of the nuclease was stopped by addition of 4 mM EGTA and by rapid cooling. Translation mixtures contained 53% of treated reticulocyte lysates and were adjusted to a final concentration of 15 mM Hepes, pH 7.5, 10 mM creatine phosphate, 54 μg/ml creatine kinase, 120 mM KAc, 0.4 mM $MgCl_2$, 2 mM DTT, 80 μg/ml of calf liver tRNA, 110 μM of each of the 19 unlabelled amino acids, 18.5–37 MBq/ml of [^{35}S]methionine and 50–100 μg/ml of viral RNA.

Neither $CaCl_2$ nor EGTA was added when the RNA was translated in untreated reticulocyte lysates except when specifically mentioned. All incubations were performed at 30°C. Samples were analyzed by polyacrylamide gel electrophoresis as described [8].

3. RESULTS

3.1. *Stability of TBRV RNA-2 translation products in reticulocyte lysates*

In a previous paper we showed that TBRV RNA-2 induces the synthesis of a 150 kDa protein (corresponding to its entire coding capacity) when incubated in a reticulocyte lysate [8]. Here we studied the kinetic of synthesis of this protein when the RNA was incubated either in untreated (fig.1A,a) or in micrococcal nuclease-treated reticulocyte lysates (fig.1A,b,c,d). The synthesis of the 150 kDa protein always reached a maximal yield after 1 h of incubation, but seemed to be degraded upon longer incubation periods. The most extensive degradation of the 150 kDa protein was observed in S nuclease-treated lysates in which a prominent 50 kDa protein, lesser amounts of 38–40 kDa proteins (these proteins are not resolved on all gels and will be called 40 kDa proteins hereafter), and a protein of about 100 kDa appeared after 1–5 h. The translation pattern of TBRV RNA-2 in B nuclease-treated lysates resembles the one obtained in S nuclease-treated lysates, although less of the smaller M_r proteins

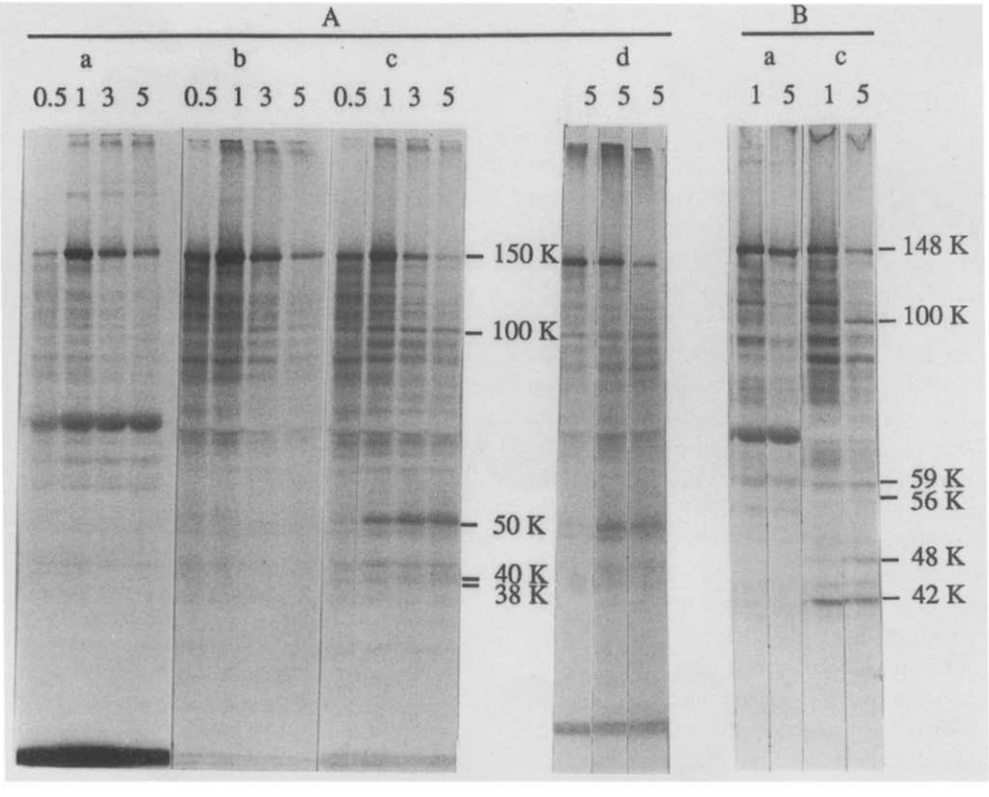

Fig.1. Kinetics of appearance of [^{35}S]methionine labelled products of TBRV (A) or GCMV (B) RNA-2 in reticulocyte lysates. Aliquots (8 μl) were withdrawn from the reaction mixture at the times indicated (h) and analyzed by electrophoresis on an 8% polyacrylamide gel. RNAs were incubated in an untreated reticulocyte lysate (a), or in a lysate pretreated with 10 μg/ml micrococcal nuclease from Worthington (b), Sigma (c) or with 5, 10 and 20 μg/ml nuclease, respectively, from Boehringer (d).

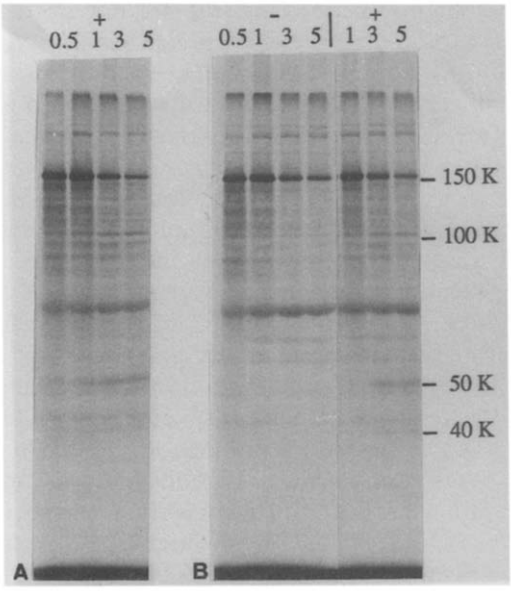

Fig.2. Time course of TBRV RNA-2 products in untreated reticulocyte lysates. (A) Micrococcal nuclease (5 μg/ml) from Sigma and EGTA (3 mM) were added to the medium at time 0. (B) Translation of TBRV RNA-2 started in an untreated reticulocyte lysate. After 45 min samples were divided in 2 aliquots and were further incubated in normal conditions (−) or supplemented with S micrococcal nuclease (5 μg/ml) and unlabelled methionine (3 mM) (+).

were obtained. Similar patterns were obtained by using 2–3 times higher concentrations of B as S nuclease (fig.1A,d). However, doubling the concentrations of W nuclease did not result in increased production of small proteins (not shown).

GCMV-like TBRV is a nepovirus and both RNAs of this virus have been sequenced (Candresse, T. and Le Gall, O., personal communication). GCMV RNA-2 codes for a 148 kDa protein. This protein shares 60% of its amino acid sequence with the TBRV RNA-2 150 kDa encoded protein. Fig.1B shows that the 148 kDa polyprotein of GCMV is susceptible to the proteolytic activity of S nuclease and proteins of 59, 48 and 42 kDa appeared.

3.2. *Evidence for a proteolytic activity present in the micrococcal nuclease*

A possible explanation for the appearance of the 100, 50 and 40 kDa proteins, in treated lysates programmed with TBRV RNA-2, is that a residual nuclease activity is able to produce RNA fragments which could be translated into small proteins. However, omission of the $CaCl_2$ during the treatment of the lysate by S nuclease, which resulted in no degradation of endogenous mRNAs, as shown

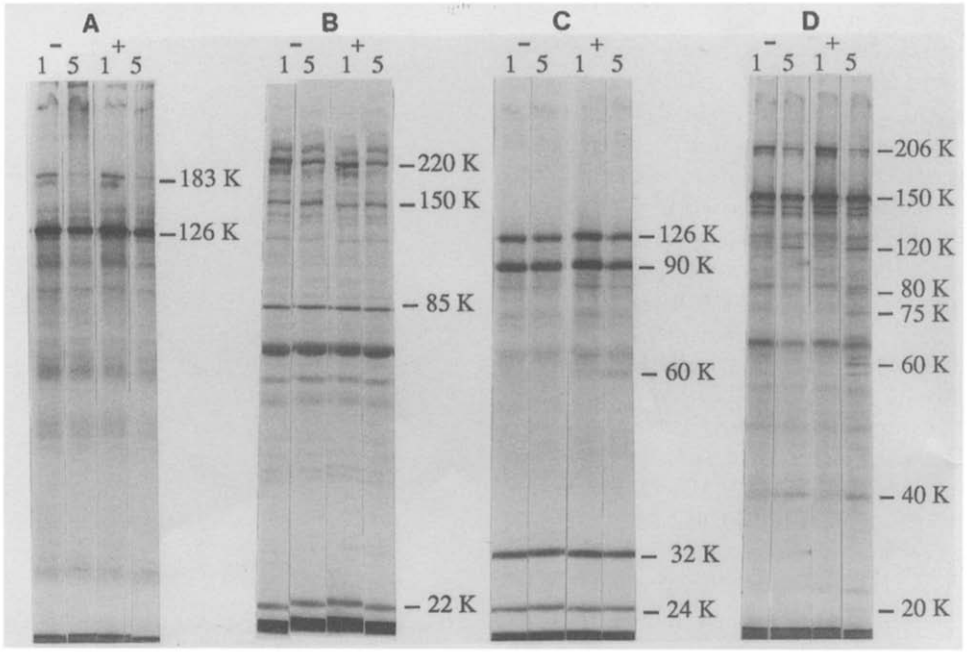

Fig.3. Comparison of translation products of TMV (A), BNYVV (B), AlMV (C) and TYMV (D) RNAs in an untreated reticulocyte lysate (−) or supplemented with S micrococcal nuclease (5 μg/ml) and unlabelled methionine (3 mM) after 45 min of incubation (+).

by efficient translation of globin (fig.2A), did not diminish the formation of the 100, 50 and 40 kDa proteins.

In a second experiment translation of RNA-2 was started in an untreated lysate and nuclease was added after 45 min when the 150 kDa protein was already synthesized. At the same time the medium was supplemented with a 2000-fold excess of unlabelled methionine to prevent labelling of newly synthesized proteins. Therefore, the small proteins which appeared soon after the addition of the nuclease, and which increased in abundance during further incubation (fig.2B), arose by cleavage of the 150 kDa protein and were not newly synthesized.

Similar experiments were carried out with TBRV RNA-1 and GCMV RNA-1 which both encode a protein of 250 kDa ([3], and Candresse, T. and Le Gall, O., personal communication). These proteins are translated in the reticulocyte lysate during the first hour of incubation, then proteins of lower M_r appeared in the lysates whether S nuclease was added or not. But additional proteins also occurred in the lysates supplemented with S nuclease (not shown).

3.3. *Behaviour of other viral proteins in the nuclease-treated reticulocyte lysate*

According to the literature many viral RNAs have been translated in treated reticulocyte systems [9], but the appearance of undesired proteins was never pointed out. Since the source of nuclease used was generally not indicated, we could not decide if the sensitivity of translation products of TBRV and GCMV RNAs towards the 'nuclease' was peculiar to these particular polyproteins or if unfortunately we were the only ones to use S nuclease.

Therefore we compared the translation patterns obtained with different viral RNAs in lysates containing nuclease or not, with those already published. Fig.3 shows that the M_r of the proteins synthesized by TMV RNA (183–126 kDa) and the BNYVV RNA-1 (220–150 kDa) and RNA-2 (85–22 kDa) were in agreement with those reported [7,10,11], and that the proteins were largely undegraded during 5 h of incubation, no new bands were produced in the presence or absence of nuclease. Also the four proteins of 126, 90, 32 and 24 kDa induced by RNA-1–4 of AlMV, respectively, described in [12] are not degraded after addition of the nuclease although a faint band of approximately 60 kDa did appear, presumably by cleavage of the 126 or 90 kDa protein; however, no evident counterpart to these proteins was detected (fig.3). With TYMV RNA in both incubation conditions we found the 200 kDa protein corresponding to translation of the largest ORF, as well as the 150 and 120 kDa proteins which could be generated by cleavage of the 200 kDa protein [13]; also an approximately 40 kDa protein is produced together with the 120 kDa protein. Surprisingly no protein of about 78 kDa described earlier as the counterpart (to the 200 kDa) of the 120 kDa protein was visible in the lysates when no nuclease was added. Again, other translation products differed when nuclease was added in the incubation medium and especially proteins of M_r of about 20, 60, 75 and 80 kDa (fig.3) were produced after addition of S nuclease.

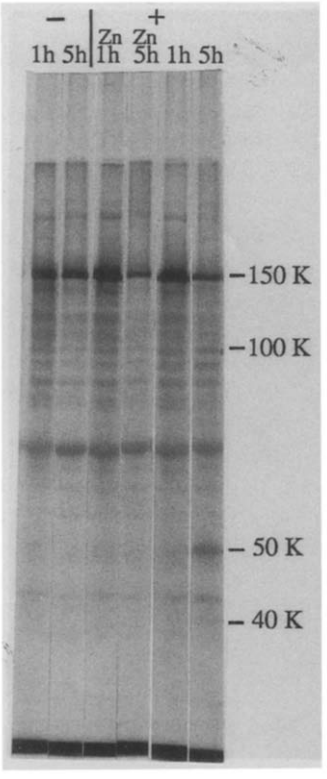

Fig.4. Translation of TBRV RNA-2 in untreated reticulocyte lysates; control samples (–), addition of S micrococcal nuclease (5 μg/ml) at 45 min (+), addition of $ZnCl_2$ (2 mM) at 20 min (Zn).

3.4. *Attempts to inhibit proteolytic cleavage*

Zinc ions are able to inhibit viral protease activity [14–16]. Therefore we examined the effect of Zn^{2+} on the proteolytic activity of the S nuclease.

Fig.4 shows that the presence of 2 mM $ZnCl_2$ in the translation mixture prevents cleavage of the 150 kDa protein into the 100 and 50 kDa proteins produced by addition of S nuclease in the medium.

4. DISCUSSION

Translation products of several virus mRNA were slightly degraded when incubated for more than 1 h in reticulocyte lysates; this may be due to diverse protease activities shown to be contained in reticulocyte lysates [17]. However, in addition to this degradation we detected a more 'specific' activity attributable to the micrococcal nuclease used to render the lysates mRNA dependent. This activity resembled a virus-coded protease, in that cleavages occurred in a limited number and only when the largest proteins were almost made, and that these were inhibited by the presence of Zn^{2+} in the medium.

Surprisingly among the translation products studied, the polyproteins translated from the RNAs of TYMV, TBRV and GCMV which undergo processing by a virus-coded protease ([3,13] and Candresse, T. and Le Gall, O., personal communication), were the most sensitive towards the proteolytic activity of the S or B micrococcal nuclease. Proteins encoded by AlMV RNAs were only slightly sensitive and those of TMV and BNYVV RNAs remained uncleaved. We do not know if this is pure coincidence, or if the regions accessible to the proteolytic activity of the nuclease and to the viral protease share similar features. Of course further sensitive polyproteins should be analyzed, and the cleaved proteins have to be localized within the polyproteins from which they originate, to define possible common characteristics of the regions concerned with the cleavage.

In conclusion, it is worth underlining that any cleavage of a viral polyprotein in an in vitro protein synthesizing system must be regarded with precaution before it can be ascribed to a virus-coded protease.

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