Initiation of protein synthesis in cell-free extracts of *Tetrahymena pyriformis*

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(Received 26 January 1981/Accepted 24 February 1981)

Saturating concentrations of the initiation-specific inhibitors poly(I) and T-2 toxin inhibit protein synthesis by over 35% and cause ribosome 'run-off' from the polyribosomes. The elongation-specific inhibitor cycloheximide totally prevents protein synthesis and 'freezes' the ribosomes in the pattern of unincubated controls. These results prove that our *Tetrahymena* extracts are capable of protein-synthesis initiation, a conclusion which is confirmed by a 30% inhibition of synthesis by the mRNA cap analogue, 7-methylguanosine 5'-monophosphate.

Although cell-free protein-synthesis systems prepared from the protozoan *Tetrahymena pyriformis* have been reported in the literature for some years, critical analysis of the results shows that the protein synthesis obtained is almost entirely due to chain elongation rather than initiation events *de novo*, because inefficient 'run-off' systems or extracts primed with artificial mRNA, such as poly(U), were used (Mager & Lipmann, 1958; Conklin & Chou, 1971; Allen & Suyama, 1972). However, we have described the preparation and partial characterization of a highly active cell-free protein-synthesizing system from this organism (David & Smith, 1981). It is important to establish whether these cell-free preparations are capable of initiating new rounds of protein synthesis, because of the potentially important use of *Tetrahymena* extracts as model systems for work on pathogenic protozoa. In the present paper we report data which do indeed show a positive initiation capacity in the postmitochondrial-supernatant extracts.

Materials and methods

Materials

Proteose-peptone and yeast extract were purchased from Difco (Detroit, MI, U.S.A.). Baker's-yeast tRNA, cycloheximide, poly(I) and 7-methylguanosine 5'-monophosphate (m^7 -GMP) were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). All radioactive chemicals were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). T-2 toxin was a gift from Dr. M. Cannon, King's College, University of London.

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Preparation of *Tetrahymena* postmitochondrial extracts and cell-free protein-synthesis incubations

Exponential-phase cultures of *Tetrahymena* were harvested and used for preparing the postmitochondrial extracts as described previously (David & Smith, 1981, in the section on 'Final procedures'), except that the preparation buffer used comprised 25 mM-KCl, 7 mM-magnesium acetate, 1 mM-dithiothreitol, 0.1 mM-EDTA, 1.5 mM-EGTA and 10% (v/v) glycerol made up in 20 mM-triethanolamine/HCl buffer, pH 7.4.

Protein-synthesis assays were performed exactly as described previously (David & Smith, 1981), except that the final incubation volume was decreased to 0.05 ml and the labelled amino acid mixture used was composed of [3 H]phenylalanine plus [3 H]leucine.

Analysis of polyribosome profiles on sucrose gradients

The initiation of protein synthesis in *Tetrahymena* S-40 extracts was determined by performing sucrose-gradient-analysis assays. Here, 0.2 ml incubations were made up essentially as described above except that all 20 protein amino acids were unlabelled and were present at a concentration of 10 μ M and the mixtures contained 0.1 ml of Sephadex-G-25-filtered S-40 extract (adjusted to contain 40 A_{260} units/ml). The assays were incubated at 20°C for 10 min, and were terminated by rapid cooling in an ice bath before layering on to linear 5.2 ml 10–40% (w/v) sucrose gradients made up in S-40-extract-preparation buffer but without 1.5 mM-EGTA. Centrifugation was at 45 000 rev./min for 55 min and 2°C in a 6 $\times$ 5.5 ml swing-out rotor of an MSE 65 Mk. II ultracentrifuge. Gradients were eluted by upward displacement with 60%

sucrose into a flow cell of 0.1 cm path length mounted in a Pye Unicam SP.8-100 automatic recording spectrophotometer set at a wavelength of 260 nm.

Preparation of protein-synthesis inhibitors

Poly(I) solutions (made up in distilled water) were heated to 90°C and cooled immediately before use.

T-2 toxin was dissolved in 50% (v/v) dimethyl sulphoxide. In all experiments controls were included at equivalent dimethyl sulphoxide concentrations to those used in reaction mixtures containing T-2 toxin.

Results and discussion

A comparison of the kinetics of protein synthesis in our *Tetrahymena* S-40 extracts in the presence of

inhibitors which specifically prevent initiation or elongation events in mammalian cell-free protein-synthesis systems is shown in Fig. 1(a). Both poly(I) (Pain, 1973), at a saturating concentration of 900 µg/ml (Fig. 1b), and T-2 toxin (Cannon *et al.*, 1976), at a saturating concentration of 30 µg/ml (Fig. 1c), gave the expected amino acid-incorporation kinetics in our S-40 extracts when initiation is selectively inhibited (Fig. 1a), i.e. complete cessation of synthesis occurred only after a lag of several minutes, during which time incorporation paralleled that in an uninhibited control. Both poly(I) (Fig. 1b) and T-2 toxin (Fig. 1c) (when the dimethyl sulphoxide blank values had been subtracted) inhibited synthesis by over 35%.

In contrast, the addition of the elongation inhibitor cycloheximide, at the saturating concentration of 100 µg/ml (Fig. 1d), caused immediate cessation of

Fig. 1. Inhibition of protein synthesis by poly(I), T-2 toxin and cycloheximide

Incorporation mixtures were prepared and 50 µl samples were treated as described in the Materials and methods section. (a) Time course of protein-synthesis inhibition: ●, untreated control; ▲, +poly(I) (900 µg/ml); ■, +T-2 toxin (30 µg/ml); ○, +cycloheximide (100 µg/ml). (b)–(d) Inhibition of protein synthesis by various concentrations of (b) poly(I), (c) T-2 toxin and (d) cycloheximide: ●, incorporation; ○, percentage inhibition. All inhibitors were added at zero time, and 50 µl samples were removed at the times indicated. Each value is the mean of two determinations.

Fig. 2. Effect of poly(I), T-2 toxin and cycloheximide on polyribosome profiles from *Tetrahymena* postmitochondrial extracts

Incorporation mixtures were prepared as described in the Materials and methods section and incubated at 20°C. All inhibitors were added at zero time and samples for sucrose-gradient analysis were removed after 0 and 10 min incubation. (a) Control at zero time; (b) control, 10 min incubation; (c) +poly(I) (900 µg/ml), 10 min incubation; (d) +T-2 toxin (30 µg/ml), 10 min incubation; (e) +cycloheximide (100 µg/ml), 10 min incubation. The percentage of polyribosomes in each incubation was calculated by weighing a cut-out tracing of the profiles. The values obtained were: (a) 80%, (b) 75%, (c) 57%, (d) 59%, (e) 79%.

protein synthesis (Fig. 1a) and hence over 95% inhibition of amino acid incorporation (Fig. 1d).

The effects of these inhibitors on the polyribosome distribution in an S-40 extract actively synthesizing protein are shown in Fig. 2. In the incubations containing poly(I) (900 µg/ml) (Fig. 2c) and T-2 toxin (30 µg/ml) (Fig. 2d) there is clear evidence of ribosome 'run-off' compared with the profiles from uninhibited (Fig. 2a) and 10 min-incubated but uninhibited (Fig. 2b) control extracts. Thus the profiles obtained with the initiation-specific inhibitors show a loss of the very-high-molecular-weight polyribosomes at the bottom of the sucrose gradients. This loss is coupled with an increase in the proportion of smaller polyribosomes and with an increase in the concentration of the 80S monomeric ribosomes at the very top of the gradient.

The profile obtained after a 10 min incubation in the presence of cycloheximide (100 µg/ml) (Fig. 2e) shows substantial identity with the zero-time control (Fig. 2a), as would be expected from an inhibitor that 'freezes' ribosome movement along the mRNA by blocking elongation events.

As an additional test of the initiation capacity of *Tetrahymena* S-40 extracts, we examined the effect of the cap analogue m⁷-GMP on cell-free protein synthesis. Structural analogues of the 5'-terminus cap structure of eukaryotic mRNA, such as m⁷-GMP, have been shown to inhibit translation of capped mRNA, but to have little or no effect on the translation of non-capped mRNA (Canaani *et al.*, 1976; Hickey *et al.*, 1976), although Kaempfer *et al.* (1978) have reported inhibition of translation with

Fig. 3. Inhibition of protein synthesis by 7-methyl-guanosine 5'-monophosphate

Incorporation mixtures were prepared as described in the Materials and methods section. 7-Methyl-guanosine 5'-monophosphate was present at the concentrations indicated on the graph. The Sephadex-G-25-filtered S-40 extract contained 57 A₂₆₀ units/ml. ●, Incorporation; ○, percentage inhibition. Each value is the mean of two determinations.

non-capped mRNA by very high concentrations of these cap analogues. Whatever the exact nature of the m⁷-GMP inhibition, a positive effect when they are included in cell-free protein-synthesizing extracts will be obtained only as a result of the inhibition of initiation and not by the inhibition of either elongation or termination events.

Fig. 3 shows the effect of increasing concentrations of m⁷-GMP on the protein-synthetic activity

of our S-40 extracts (solid circles). Incorporation was inhibited by about 30% at a concentration of $1.2\text{mM-m}^7\text{-GMP}$ (open circles). This degree of inhibition is almost identical with that reported for the experiments with poly(I) (Fig. 1c) and T-2 toxin (Fig. 1d). Since the $\text{m}^7\text{-GMP}$ concentration required to inhibit synthesis is rather high, we are still unable to confirm the possibility that *Tetrahymena* mRNA possesses a cap structure at its 5'-end.

In summary, we conclude that *Tetrahymena* cell-free extracts prepared by the method that we have previously described (David & Smith, 1981) are capable of the re-initiation of protein synthesis, although from the nature of our results we are unable to say definitively whether it is on whole or fragmented mRNA. The incorporation results obtained with poly(I), T-2 toxin and $\text{m}^7\text{-GMP}$ are in agreement and indicate initiation capacities of approx. 35% of the total protein synthesis.

Therefore, as far as we are aware these results comprise the first detailed report on the initiation of protein synthesis in cell-free systems prepared from single-celled protozoa, and as such are extremely

important to workers interested in using these organisms as model systems to study protein synthesis in the clinically important pathogenic protozoa.

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