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NUCLEASE ACTIVITY IN PREPARATIONS OF CREATINE PHOSPHOKINASE: EFFECT ON mRNA STABILITY

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Summary: Creatine phosphokinase is used to generate ATP with creatine phosphate for in vitro protein synthesis. Some preparations of this enzyme contain nuclease activity, which can be demonstrated by a sensitive assay of the cleavage of poly(A)-containing RNA. These preparations of creatine phosphokinase support protein synthesis poorly in a cell-free system prepared from HeLa cells. Poly(A)-containing RNA is quite stable in this cell-free system when the phosphorylated sugar fructose 1,6-bisphosphate with no addition of enzyme is used to generate ATP.

An ATP-generating system is required to support in vitro protein synthesis with mammalian cell extracts (1). We have previously used creatine phosphokinase to generate ATP from creatine phosphate with good results (2). However, we have recently noticed that different preparations of creatine phosphokinase support protein synthesis by mammalian cell extracts to a variable extent. This led us to suspect that commercial preparations of creatine phosphokinase may be contaminated with nucleases. We report in this communication that nuclease activity can be demonstrated in preparations of creatine phosphokinase by a very sensitive assay of mRNA integrity.

Methods: HeLa cells were grown as previously described (3). Extracts were prepared from cells washed and homogenized as indicated before (2). Hemin was added to 50 μM final concentration to the cell extracts.

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HeLa cell extracts were incubated as described (3) with an energy-generating system consisting of creatine phosphate and creatine phosphokinase. All the incubations contained 50 µM unlabeled amino acids minus lysine, 0.1 mCi/ml of [^{3}H]lysine (40 Ci/mmol) and other additions, as indicated in the figure legends. The incubations were carried out at 30°. Five-µl aliquots of the incubations were processed for counting as previously described (2).

To label mRNA, exponentially growing cultures of HeLa cells were harvested and resuspended at 2 x 10^6 cells/ml. Actinomycin D was added to 0.04 µg/ml and 20 µCi/ml of [^{3}H]uridine (25 Ci/mmol) was added after 15 min. The cells were incubated 90 min at 37°, harvested by centrifugation and used to prepare an extract for cell free protein synthesis. HeLa cell extracts containing labeled RNA were incubated under conditions used for protein synthesis. The incubations contained 20 unlabeled amino acids at concentrations of 50 µM each. At the times indicated, 10 to 50 µl aliquots were removed and diluted with 1 ml of buffer containing 0.5% Na-dodecyl sulfate, 0.1 M NaCl, 1 mM EDTA, and 10 mM Tris, pH 7.5. After increasing the NaCl concentrations to 0.5 M, the poly(A)-containing mRNA was chromatographed on columns containing 200 mg of oligo(dT)-cellulose (T-3, Collaborative Research). The bound fraction was eluted with buffer lacking NaCl as previously described (4). The RNA was precipitated with 10% trichloroacetic acid after the addition of 25 µg carrier tRNA and collected on Millipore filters for counting.

Results: Protein synthesis by an HeLa cell extract is directly proportional to the amount of creatine phosphokinase added up to 30 units/ml, and little further stimulation is obtained with 60 units/ml of creatine phosphokinase (Fig. 1). This indicates that a relatively large amount of this enzyme is required for optimal protein synthesis by this cell extract. The commercial preparation of creatine phosphokinase used in this experiment was the same used in previous published work from this laboratory (3); active protein synthesis was shown to proceed for at least 45 min (2). When a different batch of creatine phosphokinase was purchased from the same supplier and used for *in vitro* protein synthesis, we noticed that protein synthesis stopped after a 15 min incubation (data not shown). This result raised the possibility that the creatine phosphokinase contained an inhibitor of protein synthesis, possibly a nuclease activity.

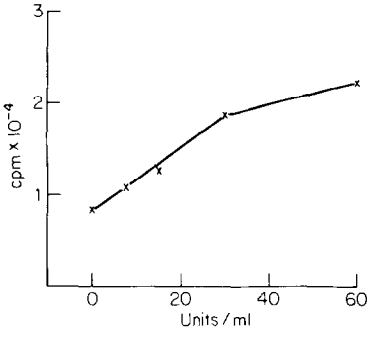

Fig. 1 - Stimulation of protein synthesis in the HeLa cell free system by different concentrations of creatine phosphokinase. The incorporation of [^{3}H]lysine into protein was assayed as previously described (2); creatine phosphokinase (SIGMA lot 83C) was added to the final concentrations indicated. Incubation was for 30 min at 30°C. Duplicate 5 µl aliquots were spotted on Whatmann 3MM filter disks and prepared for liquid scintillation counting as described (2).

In order to assay for nuclease activity under conditions of in vitro protein synthesis, we prepared an extract from HeLa cells incubated with [^{3}H]uridine in the presence of 0.04 µg/ml of Actinomycin D (see Methods). At this concentration, Actinomycin D inhibits the synthesis of ribosomal RNA and the labeled RNA present in cell extracts is predominantly mRNA and tRNA (5). The extract containing labeled RNA was incubated with creatine phosphokinase under conditions of protein synthesis and RNA degradation assayed by precipitating aliquots of the incubation with 10% trichloroacetic acid. Very little loss of RNA was observed in this way and therefore a more sensitive assay for mRNA degradation was developed. This assay is based on the quantitation of poly(A)-rich RNA present in the extract before and after incubation under conditions used for protein synthesis. The poly(A)-rich RNA is measured by binding to oligo(dT)-cellulose as previously described (4). When a poly(A)-rich RNA molecule is cleaved, only the portion

of the molecule containing poly(A) binds to oligo(dT)-cellulose. Poly(A)-rich RNA is lost at different rates in incubations containing different preparations of creatine phosphokinase (Fig. 2). The loss of poly(A)-rich RNA in a 30 min incubation varies from 8% to 74% with different commercial preparations of the enzyme (Table 1). When tested for protein synthesis, only the creatine phosphokinase preparations which cause a loss of less than 20% of the poly(A)-rich RNA, sustain amino acid incorporation longer than 15 to 20 min. We have recently reported that protein synthesis can be carried out without addition of enzymes to HeLa cell extracts by generating ATP with the phosphorylated sugar fructose 1,6-bisphosphate (6). In incubations containing this sugar and no added creatine phosphokinase, no degradation of poly(A)-rich RNA was observed (Table 1). This indicates that the HeLa cell extracts have no detectable endonuclease activity, which can degrade mRNA during in vitro protein synthesis.

Discussion: Commercial preparations of creatine phosphokinase contain variable amounts of nuclease activity, which cannot be easily detected by direct precipitation of labeled RNA. The RNA may be degraded by endonucleolytic cleavages to fragments large enough to be precipitated by 10% trichloroacetic acid. The measurement of poly(A)-rich RNA content provides, however, a much more sensitive assay of mRNA degradation. Using this method, we have shown that cleavage of mRNA takes place in incubations containing some commercial preparations of creatine phosphokinase. These preparations of creatine phosphokinase poorly support protein synthesis. This observation might explain the variability observed by different laboratories in the rate and extent of protein synthesis by

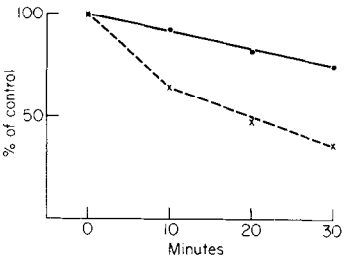

Fig. 2 - Loss of poly(A)-rich mRNA during protein synthesis with different preparations of creatine phosphokinase. HeLa cell extracts containing labeled RNA (see Methods) were used for protein synthesis. Incubations were assembled as previously described (2), but with unlabeled amino acids. At the times indicated 50 µl aliquots were removed from the incubations and chromatographed on oligo(dT)-cellulose columns as described in Methods. The percentage of poly(A)-rich RNA which binds to oligo(dT)-cellulose after incubation for the times indicated in the abscissa is shown relative to an unincubated control. The incubations contained 60 units/ml of either SIGMA lot 83C creatine phosphokinase (●—●—●), or of SIGMA lot 124C creatine phosphokinase (x--x--x).

cell free systems containing creatine phosphokinase.

The method used to measure loss of poly(A)-rich RNA is particularly sensitive to uncover few endonucleolytic breaks per molecule. The loss of poly(A)-rich RNA follows the relationship $\frac{C}{C_O} = \frac{1}{x+1}$, where C is the concentration of poly(A)-rich RNA remaining after an incubation, C_O is the initial concentration, and x are the average number of endonucleolytic breaks per RNA molecule. One random break per molecule causes the loss of 50% of the poly(A)-containing RNA. However, this procedure would not detect the loss of a few nucleotides at the 5'-terminus of mRNA, which would render the message much less active in initiating protein synthesis (7). Even with this limitation in mind, we believe that this method of measuring mRNA degradation may find important applications in the study of endonucleases present in cell extracts, which may be responsible for mRNA catabolism

TABLE I

Loss of Poly(A)-Rich RNA During Protein Synthesis with Different Preparations of Creatine Phosphokinase

Creatine phosphokinase preparations	% of poly(A)-rich RNA lost in 30 min incubation
#1	74
#2	8
#3	13
#4	27
none	0

Extracts prepared from HeLa cells incubated with [^{3}H]uridine as described in Fig. 2 were used. The energy-generating system contained 60 units/ml of creatine phosphokinase. Three different batches of this enzyme were purchased from SIGMA Chemical Co., and one batch from Boehringer Mannheim Corporation. Ten µl of the incubation mixtures were sampled before and after a 30 min incubation and the poly(A)-rich RNA determined as described under Methods. Preparation #2 of creatine phosphokinase was used for the experiment shown in Fig. 1 and in previous work from this laboratory. Preparation #1 of creatine phosphokinase failed to support protein synthesis longer than 15 min. In the experiment measuring the loss of poly(A)-rich RNA with no added creatine phosphokinase, protein synthesis was carried out with added 4 mM fructose 1,6-bisphosphate as described (6).

under some physiological conditions (8). It is extremely important for these studies that no nuclease activity should be added to the cell extracts. It may therefore be convenient to use fructose 1,6-bisphosphate or other phosphorylated sugars to generate ATP (6).

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