INCREASED TRANSLATABILITY IN A CELL-FREE SYSTEM OF RNA EXTRACTED FROM ACTINOMYCIN D-TREATED CULTURES

Gania Kessler-Icekson and David Yaffe

Department of Cell Biology

The Weizmann Institute of Science, Rehovot, Israel

Received January 17,1977

SUMMARY. — RNA extracted from myogenic cultures treated with actinomycin D was found to be more active in stimulating protein synthesis in the wheat germ cell-free system than RNA from untreated cultures. The rate of incorporation of amino acids was up to 30% higher and the synthesis of actin and of myosin light chains increased by up to 50% when RNA from actinomycin-treated cultures was used. A cell-free system product which affects the rate of translation does not seem to be involved in this phenomenon.

Actinomycin D (AMD)* is a commonly used inhibitor of RNA synthesis. In many investigations, an enhanced rate of protein synthesis was observed during the first few hours following treatment of cells with AMD (1). These included, for example, the synthesis of tyrosine aminotransferase in hepatoma cells (2) and the synthesis of ovalbumin in the chick oviduct (3). In rat primary cultures the rates of cell fusion and increase in creatine kinase activity are enhanced during the first 6 h following AMD treatment (4). An enhancement of spontaneous contraction of muscle fibers in AMD-treated cultures was also reported (5). Several mechanisms have been suggested to explain these phenomena. Among these are disappearance of shortlived mRNAs which code for repressors and the effect of AMD on the stability of mRNAs and proteins (6, 7).

We wish to report on experiments showing that purified cytoplasmic pA RNA extracted from AMD-treated myogenic cultures is more active in stimulating protein synthesis in the wheat germ cell-free system than RNA from untreated cultures.

MATERIALS AND METHODS

Cell cultures were kept and grown as described elsewhere (8). Actinomycin D (Cal-Biochem) was added to a final concentration of 4 μg/ml. Extraction of cytoplasmic RNA and isolation of polyadenylated RNA were performed according to Singer and Penman (9). Contamination by rRNA did not exceed 10%. Cell-free translation was carried out in the

*Abbreviations used: AMD, actinomycin D; CFS, cell-free system; pA RNA, polyadenylated RNA.

ISSN 0006-291X

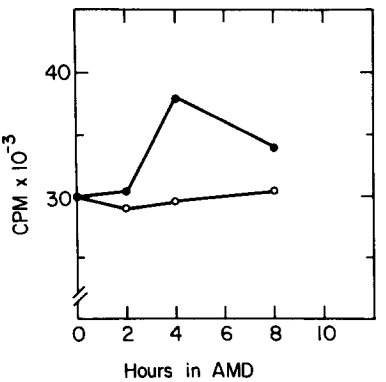

FIGURE 1. Translatability of pA RNA from cultures treated with AMD

L8 cultures at early fusion stage were taken for the experiment. AMD (4 μg/ml) was given to half of the plates at time 0; the rest of the plates were left untreated. Cultures (15 plates per point) were harvested at designated intervals and cytoplasmic pA RNA was isolated as described in Methods. Polyadenylated RNA of each point was incubated for 90 min in the wheat germ CFS at a concentration of 0.6 μg/25 μl reaction mixture. Incorporation of [^{35}S]methionine into hot TCA-precipitable material was measured in 3-μl aliquots. Ordinate, radioactivity; abscissa, time of extraction of RNA. ●—●, RNA from AMD-treated cultures; o—o, RNA from untreated cultures.

wheat germ extract according to Roberts and Paterson (10), except that KCl was replaced by K acetate (120 mM) and no exogenous tRNA was added. [^{35}S]methionine (>250 Ci/mmol) was purchased from Amersham, U.K. Analysis of cell-free products was done by electrophoresis on polyacrylamide-SDS slab gels polymerized at a gradient of 10–20% (11, 12). The gels were dried and exposed to x-ray film (Kodak Royal XOMAT RP-54). Radioactivity in each band was measured by scanning the radioautogram at 500 nm, removing the peaks under investigation from the chart paper, and weighing them. The density of the bands measured in this way was found to be linearly correlated with the cpm per band. The results are given in arbitrary units.

RESULTS

Polyadenylated RNA extracted from myogenic cell cultures directs in the wheat germ CFS the synthesis of many polypeptides which form discrete bands on acrylamide gels. Three of these were identified as actin (13) and two myosin light chains (14, 15).

Cultures of the myogenic cell line L8 at different stages of differentiation were treated with 4 μg/ml AMD. At this concentration there is more than 95% inhibition of pA RNA synthesis (Kessler-Icekson et al., in preparation). At different times following application of AMD, cytoplasmic pA RNA was extracted and tested for its capacity to direct the synthesis of proteins in the wheat germ CFS. Equal amounts of RNA at rate-limiting

TABLE 1. The synthesis of specific polypeptides in CFS directed by pA RNA from AMD-treated and untreated cultures

	Translation activity in CFS of pA RNA extracted from:		% increase
	Untreated cultures	AMD-treated cultures	
Experiment 1			
Total incorporation (cpm/3 μl)	29,000	38,000	31
Actin (arbitrary units)	243	278	14.4
LC I "	280	373	33.2
LC II "	283	346	22.2
Experiment 2			
Total incorporation (cpm/3μl)	33,800	36,700	12
Actin (arbitrary units)	203	244	20
LC I "	651	993	52.5
LC II "	945	1490	57.7

Polyadenylated RNA was incubated in the CFS for 90 min. The translation products were analyzed as described in Methods and the values obtained for the synthesis of three proteins are given above. In experiment 1, cultures were at the stage of early fusion and were exposed to AMD for 4 h. Concentration of RNA was 24 μg/ml. In experiment 2, cultures were at late fusion stage. They were exposed to AMD for 6 h and concentration of RNA was 20 μg/ml. LC I, LC II: two light chains of myosin.

concentrations were added to each CFS reaction mixture. The results showed that RNA extracted 4—8 h after application of AMD is more active in stimulating the incorporation of amino acids into acid-insoluble material. A representative experiment is shown in Fig. 1. Similar results were obtained with RNA extracted from cultures at different developmental stages: proliferating cells, fusing cells and fully differentiated cultures containing mostly multinucleated fibers. The activity of RNA from AMD-treated cultures was always 10—30% above the control. In some experiments, this increase was observed as early as 1 h after application of AMD (Kessler-Icekson et al., in preparation).

When the CFS products were electrophoresed on polyacrylamide-SDS gels, it was found that the increased activity of RNA extracted from AMD-treated cultures is

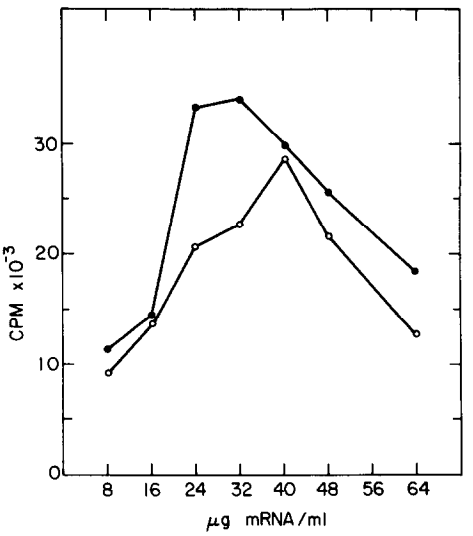

FIGURE 2. Effect of RNA concentration in the CFS on amino acid incorporation

Increasing concentrations of pA RNA extracted 4 h after administration of AMD (for details see legend to Fig. 1) from treated and untreated cultures were incubated for 90 min in the CFS. The incorporation of [^{35}S]methionine was measured for each point in 3-μl aliquots. ●—●, RNA from treated cells; o—o, RNA from untreated cells.

manifested also in the synthesis of the complete polypeptides. The relative increase in synthesis of peptides co-migrating with actin and myosin light chains, in two independent experiments, is shown in Table 1.

To further characterize the enhancing effect of AMD on the translatability of RNA, we compared the effect of increasing amounts of polyadenylated RNA extracted from AMD treated cultures and untreated cultures on the incorporation of amino acids in the CFS. At all concentrations, RNA extracted 4 h following treatment of the cells with AMD had higher translation activity. The biggest difference (30%) was observed close to the optimal concentration of RNA (25—40 μg/ml). Thus, the maximal activity of the CFS directed by RNA from AMD-treated cultures is larger than that of CFS directed by RNA from untreated cultures. The results suggest also that the maximal incorporation obtained in CFS directed by RNA from AMD-treated cultures is achieved at a lower concentration of RNA than by RNA from control cultures.

These results indicate a more efficient utilization of RNA from AMD-treated cultures for protein synthesis. Such effect could be due either to the presence of some shortlived inhibitory RNA in the control preparation which disappears after AMD treat-

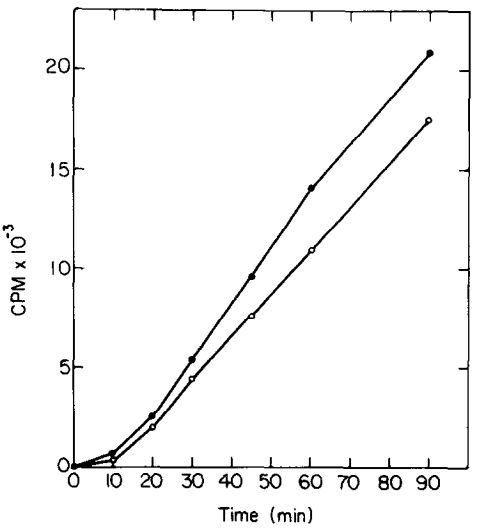

FIGURE 3. Polyadenylated RNA extracted 4 h after administration of AMD (see legend to Fig. 1) from treated and untreated cultures was incubated in the CFS at a concentration of 24 μg/ml. At different times, aliquots of 3 μl were removed to monitor the incorporation of [^{35}S]methionine. Ordinate, net cpm per 3 μl (after subtracting endogenous activity, ca. 2,000 cpm); abscissa, time from start of CFS reaction. •, RNA from AMD-treated cells; o, RNA from untreated cells.

ment or to the accumulation of enhancing factors which are stabilized in the cells in the presence of AMD. Such factors could be expected to act directly or to code in the CFS for peptides which would then influence the translation reaction (6). If the latter were the case, the initial activity in CFS directed by RNA from treated and untreated cultures would be identical and the difference in activity would build up only after a certain amount of protein was formed in the CFS. The experiment shown in Fig. 3 demonstrates that the difference in incorporation is detectable as early as 10 min after the beginning of incubation. This is almost the earliest time that any amino acid incorporation can be detected (10). Moreover, the relative difference in incorporation does not vary significantly during the entire incubation period.

Although AMD is not expected to bind to RNA, we checked for the possibility that the observed differences between the RNA preparations is due to contamination of RNA from the treated groups with AMD or to a direct effect of AMD on the RNA preparation. RNA was extracted from untreated cultures in the presence of 2 μg/ml of AMD. Before the phenol extraction the treated cytoplasm received again AMD to give 20 μg/ml and was left for 30 min at room temperature. No effect of AMD on the capacity of this RNA to stimulate incorporation of amino acids in the CFS was observed.

DISCUSSION

The present study shows that pA RNA purified from AMD-treated cultures is more active in the wheat germ CFS than RNA from untreated cultures. This higher efficiency of translation depends on some effect of AMD on the living cells and is built up during the first 1–4 h following exposure of the cells to the inhibitor. Since the difference in the activity of the RNA preparation is fully expressed less than 10 min after beginning of the incubation period (i. e., the earliest time at which incorporation of amino acids in the CFS can be measured), it is unlikely that the effect is mediated by the synthesis in the CFS of a peptide which affects translation.

A study of the kinetics of decay of cytoplasmic pA RNA in AMD-treated L8 cells indicated the existence in these cells of two major populations of molecules differing in their stability (Singer et al., in preparation): a shortlived one with a half life of ca. 2h and a more stable population with a half-life of between 17 and 54 h. Similar results were reported for other cell types (9, 16). A decrease in average size of pA RNA population extracted from AMD-treated cultures was also observed (9, 16). A causal relation between these observations and increase in translatability of RNA following treatment with AMD is possible. Differential decay of RNA species in AMD-treated cultures may affect the composition of the cytoplasmic RNA in the following ways, separately or in combination: (a) increasing the relative concentration of longlived mRNA due to disappearance of shortlived mRNA; (b) increasing the specific translation activity of the RNA in CFS as a result of decay of nontranslatable pA RNA or degradation of nontranslatable segments of mRNA; (c) disappearance of RNA which interferes with translation. The increased synthesis of actin and the two myosin light chains may be accounted for by (a). However, this should not affect the activity of the RNA in CFS as measured by incorporation of amino acids into acid-insoluble material. If the increased specific translation activity were due to (b), it would occur mainly when the concentration of RNA in the CFS was rate-limiting. However, it was observed repeatedly that the maximal translation capacity of the CFS is higher when it is directed by RNA from AMD-treated cultures. This suggests the possibility of disappearance in AMD-treated cultures of RNA which interferes with translation of the more stable mRNA, either by active suppression or by competition with the translatable mRNA for factors required for translation (e. g., shortlived mRNAs which are translated in the CFS with very low efficiency). Such a situation may explain why the differences in translatability are less prominent in low concentrations of RNA where excess of ribosomes and other CFS components may overcome part of the inhibitory effect (Fig. 2).

The myogenic L8 cells remain viable during many hours of exposure to high AMD concentrations. Therefore, these cells were convenient for long-term experiments. This may have helped us with the observations described above, yet it would be of great interest to use other cell types to study the effect of AMD treatment on RNA translatability in the wheat germ CFS as well as in other cell-free systems. Further studies are needed to understand the mechanism of the observed effect of AMD on RNA translatability, and whether or not this phenomenon is related to the enhancing effect of AMD on processes in the intact cells.

The excellent technical assistance of Mrs. Sara Neuman is acknowledged. This work was supported by grants from the Israel-U. S. Binational Science Foundation, Jerusalem; the Muscular Dystrophy Association, Inc., USA and the National Institutes of Health (Grant No. R01-GM-22767).

REFERENCES

1. Tomkins, G. M., Levinson, B. B., Baxter, J. D. and Dethlefsen, L. (1972). Nature New Biol. 239, 9—14
2. Thompson, E. B., Granner, D. K. and Tomkins, G. M. (1970). J. Mol. Biol. 54, 159—175
3. Palmiter, R. D. and Schimke, R. T. (1973). J. Biol. Chem. 248, 1502-1512
4. Yaffe, D. and Dym, H. (1972). Cold Spring Harbor Symp. Quant. Biol. 37, 543—548
5. Yaffe, D. and Feldman, M. (1964). Develop. Biol. 9, 347-366
6. Tomkins, G. M., Gelehrter, T. D., Granner, D., Martin, D., Samuels, H. H. and Thompson, E. B. (1969). Science 166, 1474—1480
7. Steinberg, R. A., Levinon, B. B. and Tomkins, G. M. (1975). Cell 5, 29—35
8. Yaffe, D. (1973). In: "Tissue Culture, Methods and Applications," eds. Kruse, P. F. and Patterson, M. K., New York, Academic Press, pp. 106—114
9. Singer, R. H. and Penman, S. (1973). J. Mol. Biol. 78, 321—334
10. Roberts, B. F. and Paterson, B. M. (1973). Proc. Nat. Acad. Sci. USA 70, 2330—2334
11. Laemmli, U. K. (1970). Nature, 227, 680—685
12. Maizel, J. V. (1971). In: "Methods in Virology," eds. Maramoroscu, K. and Koprowski, H., New York: Academic Press, Vol. V, pp. 179—246
13. Paterson, B. M., Roberts, B. E. and Yaffe, D. (1974). Proc. Nat. Acad. Sci. USA 71, 4467—4471
14. Yaffe, D., Yablonka, Z., Kessler, G. and Dym, H. (1975). Proc. 10th FEBS Meeting, North-Holland/Elsevier, pp. 313—323
15. Yablonka, Z. and Yaffe, D. (1976). Proc. Nat. Acad. Sci. USA, in press
16. Spradling, A., Hui, H. and Penman, S. (1975). Cell 4, 131-137