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The Capacity of Polyadenylated RNA from Myogenic Cells Treated with Actinomycin D to Direct Protein Synthesis in a Cell-Free System

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Cytoplasmic polyadenylated RNA of myogenic cells was shown to decay with biphasic kinetics, suggesting the existence of two main populations of mRNA with respect to stability. In the present study, the stability of mRNA extracted from actinomycin-D-treated cultures of a myogenic cell line was tested by its capacity to direct protein synthesis in the wheat germ cell-free system. The products were analyzed by dodecylsulphate/polyacrylamide gel electrophoresis. All major radioactive bands found in gels used for analyzing the products of the cell-free system directed by polyadenylated RNA extracted from untreated cultures were also found in similar gels containing products of RNA extracted after many hours of application of actinomycin D. The capacity to code for specific protein bands decays with a half-life ranging between 11 and 40 h. No fast-decaying translatable mRNA could be detected by this method. Instead, it was found that during the first 4–6 h following application of actinomycin D, the capacity of RNA to stimulate incorporation of amino acids into total acid-insoluble material increased by 20–30%. The synthesis of specific products increased by up to 100%. The possibility that the fast-decaying polyadenylated RNA or part of it is nontranslatable RNA is discussed.

It is now generally accepted that the great majority of mRNAs of eukaryotic cells contain a 3′ segment of poly(adenylic acid). The hybridization of this segment with oligo(dT) bound to cellulose has been used in many investigations as an easy method to isolate mRNA [1,2]. Experiments utilizing several methods of measurement in a variety of cell types (HeLa cells, Friend leukemia and a mosquito cell line) have shown that polyadenylated RNA decays in a biphasic manner [2–5]. Similar results were obtained whether or not actinomycin D was applied to block RNA synthesis [4,5]. These studies suggested the existence of two populations of mRNA with regard to stability.

In a previous study we investigated the decay of polyadenylated RNA in cultures of the myogenic line L8. During the first few days after plating, these cultures consist predominantly of proliferating mononucleated cells. After they reach confluency, proliferation decreases and a phase of rapid cell fusion starts. This results in the formation of a dense network of multinucleated fibers. Similar to primary skeletal muscle cultures, fusion of these cells is associated with initiation of, or great increase in, the synthesis of muscle-specific proteins and activity of several enzymes [6–8]. It was found that about 70% of the total cytoplasmic polyadenylated RNA synthesized in pulse-labelled mononucleated cells decays with a $t_{1/2}$ of about 2 h and the rest with a $t_{1/2}$ of 17–50 h. After formation of the multinucleated fibers, the fast-decaying population comprises only about 30% of the newly synthesized polyadenylated RNA [4]. In order to obtain some further insight into the nature of the biphasic decay of polyadenylated RNA, the stability of mRNA in the myogenic cells was investigated by assaying the capacity of RNA extracted at different times following application of actinomycin D to code for the synthesis of polypeptides in a cell-free system. Polyadenylated RNA extracted from myogenic cultures directs, in the wheat germ cell-free system, the synthesis of many polypeptides which can be separated into discrete bands by electrophoresis on polyacrylamide gels. Three of these bands have been shown to contain actin and two myosin light chains [9,10]. Thus, it is possible to assay the effect of actinomycin D on the functional stability of mRNA.

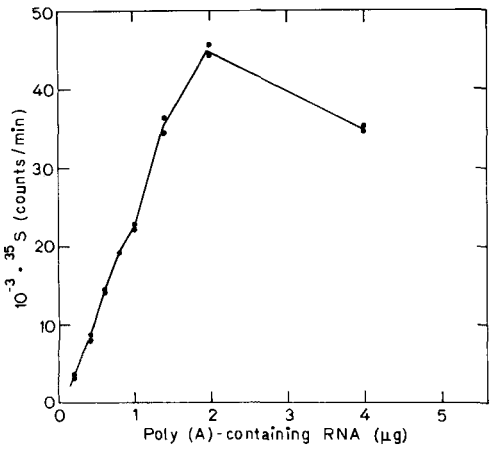

Fig. 1. *Effect of RNA concentration in the cell-free system on amino acid incorporation.* Increasing amounts of polyadenylated RNA isolated from differentiated L8 cell cultures were added to 50 µl reaction mixture of the wheat germ cell-free system. After 90 min of incubation at 22 °C, 3-µl aliquots were taken and radioactivity in material insoluble in hot trichloroacetic acid was monitored. Endogenous activity was subtracted from each point (no RNA added: 3500 counts/min)

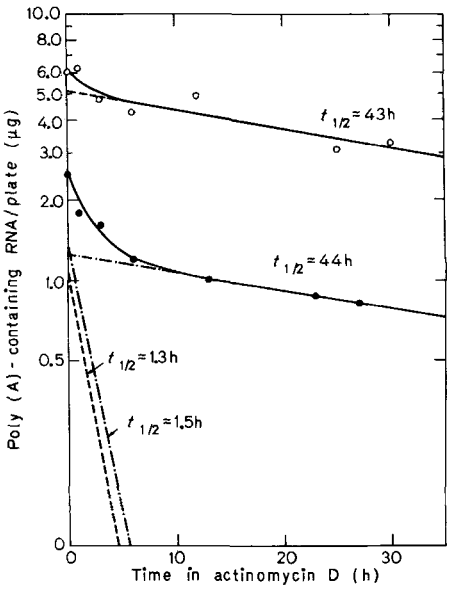

Fig. 2. *Decay of polyadenylated RNA in cultures treated with actinomycin D.* Prefusion and postfusion cultures were exposed to 4 µg/ml actinomycin D. At different times thereafter, cultures were harvested and cytoplasmic polyadenylated RNA was isolated; 10–12 prefusion (●) and 4–7 postfusion (○) plates were taken for each point. The amount of polyadenylated RNA was measured by absorbance and calculated per plate (1 A_{260} unit = 40 µg RNA/ml). The broken lines represent the decay of the two components of the curve, subtracting one from the other

MATERIALS AND METHODS

Cell Cultures

Cells of the myogenic line L8 were grown on gelatin-coated tissue-culture plates and fed with Waymouth medium (GIBCo) supplemented with 10% horse serum [11]. Unless otherwise specified, 100-mm plates were used. At the prefusion stage, cultures consisted of mononucleated cells; at the postfusion stage, over 70% of cell nuclei were located within multinucleated fibers.

Purification of Cytoplasmic Polyadenylated RNA

Extraction of cytoplasmic RNA and isolation of polyadenylated RNA was done according to Singer and Penman [2], as described in the preceding paper [4]. Contaminating rRNA species were found to comprise less than 10% of the total material binding to the oligo(dT)-cellulose (see also [2, 12]).

Cell-Free Translation of Polyadenylated RNA

The wheat germ extract (supernatant obtained by centrifugation at $30000 \times g$) was used following Roberts and Paterson [13], except that KCl was replaced by potassium acetate (120 mM) and no exogenous tRNA was added. The labelled amino acid was [^{35}S]-methionine (specific activity > 250 Ci/mmol from The Radiochemical Center, Amersham, U.K.). In each 50-µl reaction, 1 µg of polyadenylated RNA was incubated at 22 °C for 90 min. In these conditions the rate of amino acid incorporation was linearly related to the amount of RNA added to the cell-free system reaction mixture (Fig. 1) [14]. All chemicals employed in the cell-free system were purchased from Boehringer.

Analysis of Cell-Free Products

Aliquots (4 µl) of the reaction mixtures containing the labelled products were analyzed on 10–20% polyacrylamide/dodecylsulphate slab gels [15, 16]. The gels were dried under vacuum and exposed to X-ray film (Kodak, Royal X-Omat, RP-54). The radioactivity present in each band was measured by scanning the radioautograms in a Gilford 2400 S spectrophotometer at 500 nm, using a 0.1×2.36-mm slit. The peaks under investigation were cut out of the recorder chart and weighed on analytical scales. Calibration experiments showed a linear correlation between the radioactivity in a band and the intensity of blackening of the film.

Plotting of Decay Curves

Monophasic curves were evaluated by the least-square-fit method. Biphasic curves were evaluated by biexponential least-square fit, kindly programmed for us by Dr G. Yagil.

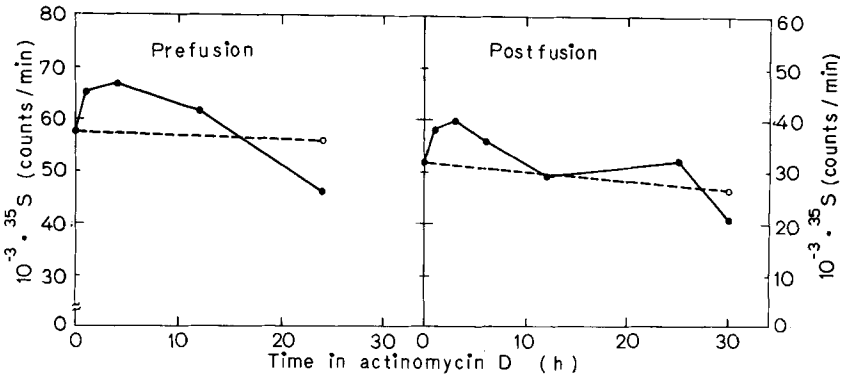

Fig. 3. *Effect of actinomycin D treatment on the capacity of polyadenylated RNA to stimulate incorporation of [^{35}S]methionine in the wheat germ cell-free system.* Prefusion and postfusion cultures were treated with 4 μg/ml actinomycin D (●—— ●) or untreated (○----○); at different times thereafter, cultures were harvested and cytoplasmic polyadenylated RNA was purified; 1 μg of the polyadenylated RNA extracted at each time point was incubated in 50 μl reaction mixture of the wheat germ cell-free system, as described in Methods. The incorporation of [^{35}S]methionine into material precipitable in hot trichloroacetic acid was measured in 3-μl aliquots and monitored in toluene

RESULTS

Decay of Polyadenylated RNA in Actinomycin-D-Treated Cells

As shown elsewhere, L8 cultures survive in the presence of actinomycin D for over 30 h, without significant loss of cells. Likewise there is no significant decrease in amount of DNA or protein during that period [4]. Therefore, in actinomycin-D-treated groups, expression of the results per plate is similar to expressing them per cell.

As previously reported [4], polyadenylated RNA extracted from whole cytoplasm of L8 cells at different times following treatment with actinomycin D decays in a biphasic pattern, indicating the existence of two populations of molecules, differing in their stability. At both stages of differentiation (prefusion and postfusion) the fast-decaying population showed a half-life ($t_{1/2}$) of 1.5 h and the slow-decaying a $t_{1/2}$ of 40–44 h (Fig. 2). However, the proportional amount of the more stable population increased during cell differentiation from 50% to 83% (estimated from extrapolation of each slope to time zero).

Stimulation of Incorporation of Amino Acids in a Cell-free System by RNA Extracted from Actinomycin-D-Treated Cells

Polyadenylated RNA extracted from cells at different times after administration of actinomycin D was tested for its ability to direct the synthesis of proteins in the wheat germ cell-free system. Equal amounts of polyadenylated RNA were incubated in the wheat germ extract. The amount of polyadenylated RNA (1 μg/50 μl) was rate-limiting (Fig. 1). The results are shown in Fig. 3. It was found that polyadenylated RNA extracted during the first few hours of exposure to actinomycin D was more active in stimulating amino acid incorporation than polyadenylated RNA extracted from untreated cells. The most active RNA was that extracted 4–6 h after addition of actinomycin D. The increase in translatability was consistently observed in many experiments. This phenomenon seemed to be independent of developmental stage, as it occurred similarly with RNA extracted from cultures at the prefusion and postfusion stages. After this phase of increase, a phase of moderate decrease in the translation activity is observed, decaying with a $t_{1/2}$ of approximately 30 h.

In order to express the changes in capacity to stimulate incorporation of amino acids in the cell-free system per culture, the values shown in Fig. 3 were multiplied by the amount of polyadenylated RNA per plate extracted at each time point. As can be seen from Fig. 4, in both prefusion and postfusion cultures the decay in the capacity to stimulate protein synthesis in the cell-free system seems to be monophasic, with similar half-life times: 16 h for prefusion and 19 h for postfusion cultures.

Analysis of Cell-Free Products

In order to investigate the ability of RNA extracted at different times after exposure to actinomycin D to code for specific proteins, the polypeptides synthesized in the experiment described in Fig. 3 were separated on polyacrylamide/dodecylsulphate gels. The radioautograms of the gels exposed to X-ray film are shown in Fig. 5. All the detectable bands formed by products of the cell-free system stimulated by RNA extracted from untreated cultures are formed also by the products of cell-free system stimulated by RNA extracted from cells that were exposed to actinomycin D for 10 h or more. Thus, the fast-decaying species of polyadenylated RNA are either not translated in this cell-free system or the products are not detectable

by this method, due to their great heterogeneity. Another possibility is that the products of the two populations of polyadenylated RNA are not qualitatively distinguishable.

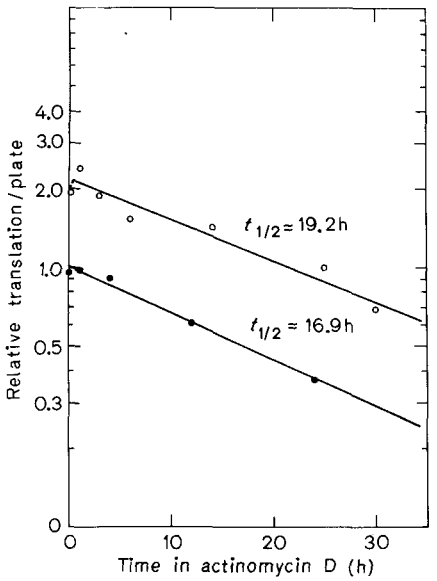

Fig. 4. *Decay of translation capacity per plate in actinomycin-D-treated cultures.* The values shown in Fig. 3 were multiplied by the amount of polyadenylated RNA obtained from one plate at each time point. The results are expressed as the relative capacity of oligo(dT)-bound RNA obtained from one culture at each time point to stimulate incorporation of [^{35}S]methionine *in vitro*. The value for untreated prefusion cultures is taken as 1. (●——●) Prefusion; (○——○) postfusion

The relative synthesis of specific polypeptides was estimated as described in Methods, by measuring the intensity with which their corresponding bands darkened the X-ray film. Fig. 6 describes the effect of actinomycin D on the synthesis, in the cell-free system, of four polypeptide bands detected on polyacrylamide gels. One of them was shown to contain actin [9] and two others to contain polypeptides with the properties of myosin light chains [10]; the fourth band is unidentified as yet and has a molecular weight of about 50000. In the present study it will be designated as protein 2.

It can be seen that the increase in translation activity of RNA extracted from actinomycin-D-treated cultures is also expressed in the synthesis of each of the four radioactive bands (as well as in the synthesis of several other bands investigated but not shown here). In all cases, the main period of increase in activity is during the first 4 – 6 h. Twice as much radioactive actin and protein 2 was translated in the cell-free system directed by polyadenylated RNA extracted from postfusion cultures after 6 h in actinomycin D as was translated in the cell-free system containing an equal amount of RNA from untreated cells. The translation of the two bands containing the light chains of myosin is increased by 50%. After this first increase, the curves describing translation for each of the peptides differ one from the other. The capacity to translate protein 2 decays faster than that for actin, while the two myosin light chain bands are translated at the elevated rate for another 20 h before decay

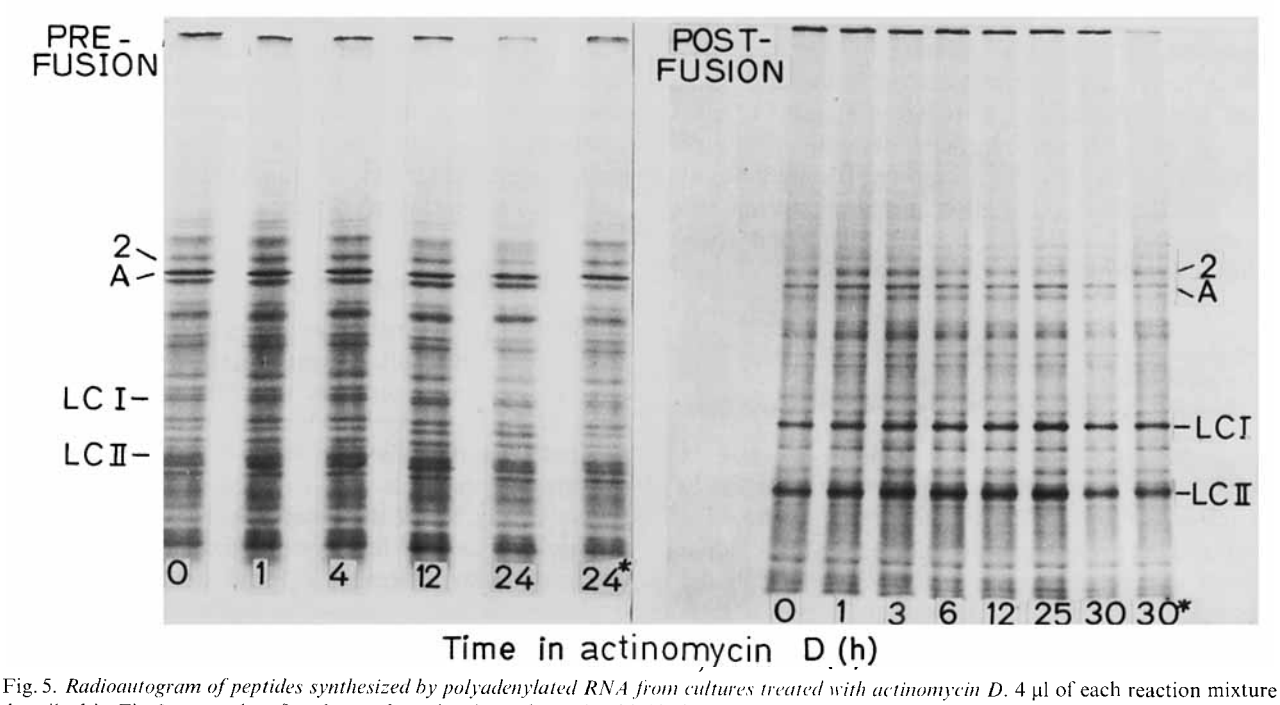

Fig. 5. *Radioautogram of peptides synthesized by polyadenylated RNA from cultures treated with actinomycin D.* 4 µl of each reaction mixture described in Fig. 3 were taken for electrophoresis on a polyacrylamide/dodecylsulphate gel. The gels were dried and exposed to X-ray film. 2 = protein 2; A = actin; LCI, LCII = myosin light chains. The asterisk denotes RNA from untreated cultures

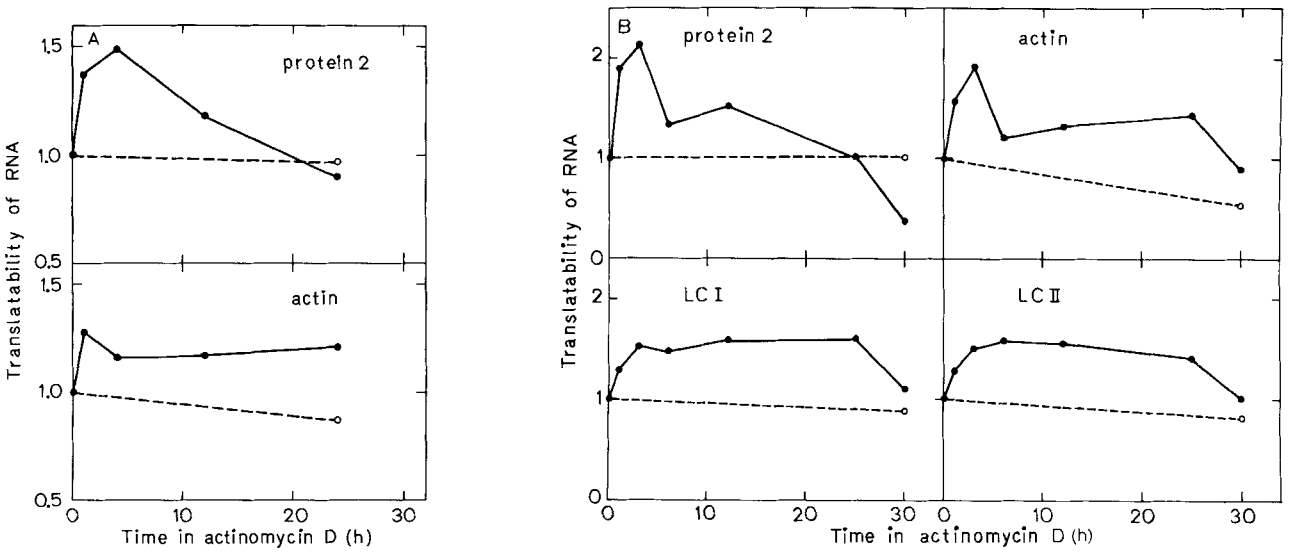

Fig. 6. *Effect of exposure of the cultures to actinomycin D on the synthesis of specific proteins in the cell-free system.* The radioautograms shown in Fig. 5 were scanned as described in Methods. The radioactivity of the marked bands is expressed in arbitrary units as the translatability of RNA. (A) Prefusion; (B) postfusion; (●—●) RNA from treated cultures; (O - -O) RNA from untreated cultures

is observed. Similar results are obtained for RNA from cultures at the prefusion and postfusion stages. (No measurable amounts of light chain were synthesized in the cell-free system directed by RNA extracted from prefusion cultures [7, 10, 17].)

In order to estimate the stability of the corresponding mRNAs as reflected by their translatability, the value describing the radioactivity of each band (directed by 1 μg of polyadenylated RNA) was multiplied by the amount of polyadenylated RNA extracted from a culture at each time point (referred to hereafter as translation capacity per plate). The results are shown in Fig. 7. The decay appears to be monophasic. The range of $t_{1/2}$ values obtained in three independent experiments for the decay in translation capacity of these proteins is shown in Table 1. In all experiments the capacity (per plate) to code for protein 2 had the shortest half-life (11 – 16 h), whereas that for the heavier light chain of myosin had the longest (30 – 40 h).

Table 1. *Range of half-lives of four different mRNAs, as measured by their translation activity in a cell-free system*
The half lives of four different mRNAs were calculated as described in Fig. 7 and the values obtained in three independent experiments are given. LCI and LCII are the light chains of myosin

Protein	M_r	$t_{1/2}$ of translatable RNA
		h
Protein 2	≈ 50000	11 – 16
Actin	42000	20 – 28
LCI	23000	30 – 40
LCII	17000	25 – 35

DISCUSSION

The observation that in a variety of cell types polyadenylated RNA decays in a biphasic manner [2 – 5] led to the suggestion that mRNA in many eukaryotic cells consists of two main populations with regard to stability. This was based on the assumption that quantitative changes in polyadenylated RNA represent changes in mRNA content. However, in investigations in which the stability of specific mRNA was measured, a monophasic decay was observed [3, 18 – 20].

In many studies, actinomycin D was used to block further RNA synthesis and the decay of protein synthesis was followed *in vivo*. Conclusions on stability of mRNA based on protein synthesis in the intact cells may be quite erroneous due to the inhibitory effects of actinomycin D on protein synthesis not related to availability of mRNA [21] and a possible effect of actinomycin D on the amino acid pool [18]. To overcome this, we attempted in the present study to measure the stability of mRNA by testing the capacity of purified polyadenylated RNA to stimulate protein synthesis in the cell-free system.

Different species of mRNA may differ in the efficiency of their translation in the cell-free system. However, it is fair to assume that at rate-limiting concentrations of RNA, differences in the relative amount of a radioactive product synthesized in identical cell-free system conditions reflects differences in the availability of the mRNA coding for these

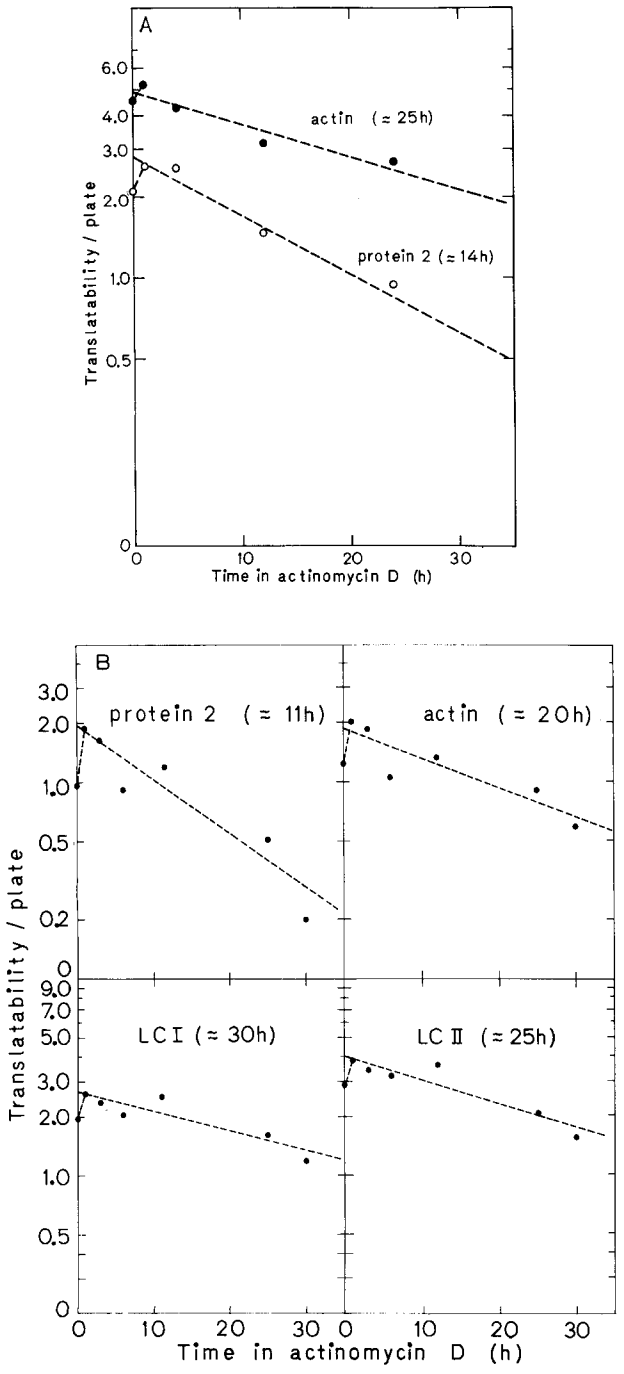

Fig. 7. *The capacity to code for specific proteins retained in actinomycin-D-treated cultures.* The amount of radioactivity obtained from 1 µg polyadenylated RNA for each band was multiplied by the amount of polyadenylated RNA extracted from the plate at each time point to give the translation capacity per plate (in arbitrary units). The times in parentheses are the half-life values. (A) Prefusion; (B) postfusion; LCI, LCII, myosin light chains

proteins. As far as can be detected on sodium dodecylsulphate gels, RNA extracted after up to 30 h of exposure of the cultures to actinomycin D and RNA from untreated cultures code for the same major polypeptides in the wheat germ cell-free system. There is no indication of fast-decaying species of translatable mRNA. On the sodium dodecylsulphate/polyacrylamide gels, the cell-free system products segregate into about 50 discrete radioactive bands, with M_r ranging from 10000–100000. (Peptides of higher molecular weight, such as the heavy chain of myosin, were not synthesized by the wheat germ extract.) The detected peptides are probably the products of the more abundant species of mRNA, whereas the products of other species, each present in small amounts, may be undetectable by the present methods. Thus, if the fast-decaying RNA consists of the mRNA species present in small amounts, then this decay will not show up on the dodecylsulphate gels (e.g. $t_{1/2}$ for the mRNA coding for tyrosine aminotransferase is in the order of 1.5 h [22]). In that case, however, one would still expect to see, parallel to polyadenylated RNA decay (Fig. 2), an initial fast decay of the capacity to stimulate incorporation of amino acids in the cell-free system, when data of Fig. 3 were expressed per plate. However, no such decay was found (Fig. 4).

A striking phenomenon observed in these experiments is the increase in translation activity of polyadenylated RNA extracted from actinomycin-D-treated cultures. This increase builds up during the first 6 h following exposure to actinomycin D and is expressed both in the overall incorporation of amino acids and in the synthesis of specific polypeptides. Since this increase takes place during the period of the fast decrease in amount of polyadenylated RNA in the cells, a causal relation between these two phenomena is suggestive. Thus, if the fast-decaying RNA consists mostly of RNA which is either not messenger or is translated in the cell-free system with low efficiency, then decay of this component will result in increase in specific translation activity of the rest of the polyadenylated RNA. It should be noted in this respect that the average size of polyadenylated RNA decreases with time in cultures treated with actinomycin D [2, 4, 5] (see also Table 1). Thus, if the larger mRNAs are less stable than the shorter ones, then polyadenylated RNA from actinomycin-D-treated cultures will contain more initiation sites than equal amounts of RNA extracted from untreated cultures.

There are, however, a few facts which suggest the possibility that the increase in efficiency of translation following treatment with actinomycin D may not merely be the arithmetic result of the decay of a nontranslatable polyadenylated RNA species, or of a change in the molarity of the translantable RNA.

a) The fast-decaying component of polyadenylated RNA from prefusion cultures is about 50% of total polyadenylated RNA. In the postfusion cultures, it is only 10–20% of total polyadenylated RNA. Yet, the increase in rate of amino acid incorporation directed by RNA from actinomycin-D-treated cultures is

about 20% in both cases. Furthermore when the products formed *in vitro* are analyzed on gels, the increase in radioactivity of specific bands in the actinomycin-treated group is up to 100% over the control. A similar increase is observed with RNA from both prefusion and postfusion actinomycin-D-treated cells.

b) Disappearance of a nontranslatable fraction or high-molecular-weight mRNA should be expected to increase the efficiency of translation of the surviving RNA but should not affect the total translation capacity per plate. However, it was found that during the first few hours, the translation capacity is higher in the actinomycin-D-treated groups, even when it is calculated per plate (Fig. 7).

These results raise the possibility that polyadenylated RNA extracted from untreated cultures contains a short-lived component which interferes with the translation of the RNA in the cell-free system [14]. The enhancing effect of actinomycin D on synthesis of a variety of proteins in intact cells has been reported (reviewed in [23]). Increase in the rate of spontaneous contractions of fibers in actinomycin-D-treated muscle cultures was also observed [24].

Whatever the functional properties of the fast-decaying RNA, since it was isolated by oligo(dT) affinity chromatography, it has to be assumed to be either polyadenylated RNA with a different stability or a non-polyadenylated RNA physically associated with polyadenylated RNA. Another possibility is that it consists of noninformational sequences in mRNA molecules which were degraded faster than the informational parts (e.g. cytoplasmic processing of mRNA).

Yet another possibility which should be considered is that the fast-decaying RNA is nuclear RNA. In all investigations in which fast-decaying polyadenylated RNA was reported, RNA was isolated from the cytoplasm after removal of the nuclei. We checked and found no ribosomal RNA precursors (45 S, 32 S) in our RNA extracts. However, leakage of smaller heterogeneous RNA cannot be entirely excluded. Such RNA would be expected to disappear quickly as a result of inhibition of new RNA synthesis, or in pulse and chase experiments.

The observed enhancement in translation activity of RNA from actimomycin-D-treated cultures complicates the quantitative determination of the decay of specific mRNA. It is also important to note that since the analysis was made on one-dimensional gels, some bands could contain more than one protein. The results however, allows us to conclude from these experiments that polyadenylated RNAs which code for the major proteins in the cell-free system are very stable and remain functional in the myogenic cell lines after many hours of treatment with actinomycin D. It seems also that, even under conditions in which the regulatory role of the nucleus is blocked by actinomycin, there are differences in stability between different mRNA species. For those proteins common to prefusion and postfusion cultures, no significant change in stability of translatable mRNA was observed following differentiation. It is important to stress, however, that the experiments were done with an established myogenic cell line. These cells appear to survive in culture in the presence of actinomycin D longer than primary skeletal muscle cultures. It is thus unsafe to draw conclusions on the stability of mRNA in primary cultures from experiments with cells of a myogenic line.

During the preparation of this manuscript it was shown that there are at least three actin isozymes, separable by isoelectric focusing. One of them is muscle-specific; its synthesis increases considerably during differentiation [25,26]. Similar results were obtained with the myogenic cell line used in the present experiments (D. Katkoff and D. Yaffe, unpublished). It would be of interest to compare the stability of mRNA coding for the different actins.

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