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Translational Capacity of Deadenylated Messenger RNA

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Summary

In order to determine whether the functional capacity of polyadenylated messenger RNA is altered when the poly(A) segment is shortened as a consequence of mRNA aging, we compared the abilities of differentially labeled new and old mRNAs to engage ribosomes. Experiments were performed using both exponentially growing mouse L cells and L cells in which the polyribosomes were temporarily disassembled as a result of a temperature shock. Our results indicate that new and old messages engage ribosomes with roughly equivalent efficiencies, suggesting, therefore, that length of poly(A) is not a determining factor for mRNA translation. We then examined the question of whether an mRNA that was originally polyadenylated could function if the Poly(A) segment were removed. This was accomplished by submitting polyadenylated mRNA to a limited digestion with a 3′-OH specific exoribonuclease, reisolating the residual polyadenylated and deadenylated mRNA, and measuring their template activities using a cell-free protein synthesizing system prepared from wheat embryos. Such experiments also failed to reveal any effect of the poly(A) on the translational capacity of the mRNA.

Introduction

The polyadenylate [poly(A)] segments located at the 3′-OH ends of most types of eucaryotic messenger RNA (mRNA) become progressively shorter in length as the messages age (Mendecki, Lee, and Brawerman, 1972; Greenberg and Perry, 1972a; Sheiness and Darnell, 1973). Indeed some messages that are polyadenylated when they are first assembled into polyribosomes may become completely devoid of poly(A) after being in the cytoplasm for times on the order of a cell generation (Murphy and Attardi, 1973; Greenberg and Perry, unpublished); and some messages, notably those coding for histones in mammalian cells (Adesnik and Darnell, 1972; Greenberg and Perry, 1972b) and some of the nonhistone messages in sea urchin embryos (Nemer, Graham, and Dubroff, personal communication), do not possess detectable poly(A) segments even when they enter the polyribosomes.

Such observations led to the speculation that poly(A) might play a role in regulating either the stability of the mRNA or its translational capacity. Recent studies of the turnover of mRNA in mammalian cells (Greenberg, 1972; Singer and Penman, 1973; Perry and Kelley, 1973; Murphy and Attardi, 1973) indicate that there is neither a progressive increase with time in the probability of decay of the polyadenylated messages nor any markedly greater susceptibility to random decay of the histone mRNAs as compared to the polyadenylated mRNA. Such findings do not readily suggest a role for poly(A) as a determinant of message stability.

The experiments reported here were performed in order to examine the second possibility, that is, whether poly(A) content influences the functional capacity of mRNA. We have made two types of evaluation of mRNA function: (1) in vivo determinations of the relative abilities of new and old mRNA to engage ribosomes in both steady state and transient conditions and (2) measurements of the template activity of polyadenylated and deadenylated mRNA in a cell-free protein synthesizing system. The results of both types of experiment indicate that deadenylation does not markedly reduce the functional capacity of mRNA.

Results and Conclusions

The first question asked was whether "new" mRNAs, that is, those that have recently emerged from the nucleus, and which contain rather uniform poly(A) segments approximately 180 nucleotides long, and "old" mRNAs, that is, those that have resided in the cytoplasmic polyribosomes for a cell generation or so, and which contain smaller, less uniform poly(A) segments, differ with respect to the efficiency with which they are translated. Such a difference in translational efficiency, if it involved a *preferential* change in either the rate of initiation or the rate of elongation, would lead to an alteration in the relative number of ribosomes that simultaneously translate a particular sized message. In this case, one would expect to find a difference in the "loading ratio" (number of ribosomes per unit length of message) for new and old mRNAs.

The characteristic loading ratios of new and old mRNAs were determined for an asynchronous culture of exponentially growing L cells, in which the polyribosome population can be considered as being in a steady state of formation and disassembly. In this experiment, illustrated in Figure 1 and Table 1, old and new mRNA were differentially tagged by incubating the cells prior to harvesting with $^{32}PO_4$ for 20 hr and ^{3}H-adenosine for 1.75 hr. From knowledge of the mean lifetime of L cell

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mRNA (Greenberg, 1972; Perry and Kelley, 1973) it is calculated that about half of the ^{32}P-labeled mRNA was in the polyribosomes for more than 10 hr, and only about 11% for 1.75 hr or less. Thus, such differential labeling should readily distinguish new and old mRNA components.

The size distribution of the polyadenylated ^{3}H and ^{32}P-labeled mRNAs isolated from small, medium, and large polyribosomes and purified by oligo dT-cellulose chromatography is very similar (Figure 1). The slightly smaller size of the old mRNA is most probably the result of the decreased lengths of the poly(A) segments (Greenberg and Perry, 1972a). It is clear from the data of Table 1 that there is no marked difference in the ratio of new to old mRNA among the three size classes of polyribosomes. This fact, also observed in HeLa cells by Singer and Penman (1973), suggests either that there is no difference in translational efficiency between new and old mRNA, or that the same ratio of initiation and elongation rate is maintained. As will be seen later, the former possibility seems more likely.

Table 1. Distribution of New and Old mRNA on Polyribosomes

Polyribosome fraction	Specific activity cpm/A260nm		
	^{3}H	^{32}P	$^{3}H/^{32}P$ (new/old)
I (small)	63,600	3,690	17.3
II (medium)	30,300	1.475	20.5
III (large)	15,050	795	18.9

Radioactivity of mRNA, purified as described in Figure 1, was assayed by adsorbing the mRNA to poly(U)-impregnated fiberglass filters (Sheldon, Jurale, and Kates, 1972), which were then dried and counted in a toluene-based scintillant. A_{260nm} values, measured on polyribosomal RNA fractions prior to passage through oligo dT-cellulose columns, represent mainly ribosomal RNA.

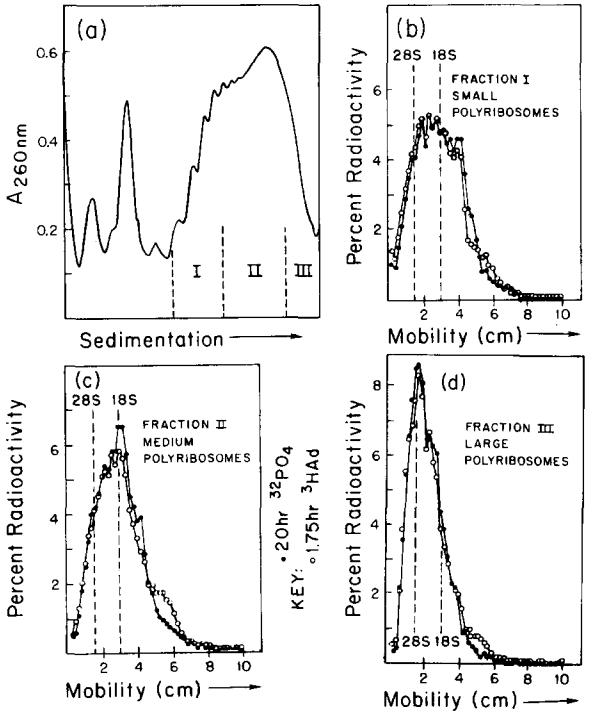

Figure 1

A 600-ml culture of L cells at an initial concentration of 1.3×10^5 cells/ml was labeled for 20 hr with 2 μC $NaH_2{}^{32}PO_4$/ml, concentrated 6-fold and incubated 1.75 hr with ^{3}H-adenosine (10 μC/ml, 0.4 μM). Cells were lysed by successive treatments with 0.05% and 0.1% Triton X-100, S16 fractions prepared, and polyribosomes isolated on 15–45% (w/w) sucrose gradients (Schochetman and Perry, 1972). The fractions containing small, medium, and large polyribosomes [I, II, and III, respectively, panel (a)] were pooled, precipitated with ethanol, redissolved, and RNA extracted with 0.5% Sarkosyl-chloroform-phenol. The RNA was passed through oligo dT-cellulose columns (Nakazato and Edmonds, 1972) and the poly(A)(+) mRNA isolated and characterized by electrophoresis on 2.7% polyacrylamide gels [panels (b)–(d)]. Electrophoresis for 2.5 hr at 5 mA/gel.

It may also be noted in Figure 1 that the small mRNAs are largely confined to the small polyribosomes, but large mRNAs are present in both small and large polyribosomes. This observation, also borne out by the higher specific activity of the small polyribosomes (Table 1), indicates that the large mRNAs are translated with a wide dispersion of loading ratios. This might occur either because the large mRNAs contain widely varying proportions of translated to nontranslated sequences, or because there is a considerable difference in the ratio of initiation to elongation for different mRNA species.

Another means of assessing the translational efficiency, particularly in regard to the initiation steps, is the ability of mRNA to re-engage ribosomes following a massive polyribosome disaggregation. Such disaggregation is induced very rapidly when cells are exposed to a brief temperature shock (McCormick and Penman, 1969; Schochetman and Perry, 1972). At 42°C the initiation process is preferentially blocked, resulting in ribosome run-off and the release of free mRNP. When the cells are returned to 37°C, their normal growth temperature, the polyribosomes rapidly reassemble, utilizing the previously released mRNP. We therefore posed the question: Will the reassembled polyribosomes contain a relative proportion of new and old mRNA which is different from that in the original polyribosomes?

The results of an experiment designed to answer this question are given in Table 2. In this case, old mRNA was labeled by incubating the cells for 2 hr with ^{3}H-uridine followed by a 21-hr chase with unlabeled uridine and new mRNA was labeled by incubating the cells for 1 hr with ^{14}C-uridine. After the incubation with ^{14}C-uridine, the cells were given a 20-min incubation with actinomycin D in order to deplete as much as possible the pools of pre-mRNA, exposed briefly to the elevated temperature and then allowed to recover at normal temperature.

Table 2. Distribution of New and Old mRNA on Polyribosomes Reassembled after Heat Shock

Source of polyribosomes	Quantity of mRNA (cpm)		
	^{14}C (new)	^{3}H (old)	$^{14}C/^{3}H$ (new/old)
Control (unshocked) cells	6,210	5,590	1.11
Shocked cells after recovery	4,620	3,660	1.26

L cells were sequentially treated as follows: a 2-hr incubation with ^{3}H-uridine; a 21-hr chase with unlabeled uridine; a 1-hr incubation with ^{14}C-uridine; and a 20-min incubation with 2 μg/ml actinomycin. A portion of the cells was further incubated for 34 min at 37°C and then harvested; this served as the control (unshocked) sample. An equivalent portion of cells was incubated for 12 min at 42°C, a further 22 min at 37°C, and then harvested; this portion served as the "recovered" sample. The extent of polyribosome disassembly due to the temperature shock was monitored on a third sample of cells harvested after the 42°C incubation. It was found that about 64% of the polyribosomes were converted to monoribosomes at 42°C, and that approximately 85% of these ribosomes were reassembled into polyribosomes after the 37°C recovery period. RNA was extracted from the polyribosomes of the control and "recovered" samples, and the mRNA purified by chromatography on oligo dT-cellulose columns. Radioactivity was assayed by adsorbing the mRNA to poly(U)-impregnated fiberglass filters as in Table 1.

It is evident from these data that the ratio of new/old mRNA in the reassembled polyribosomes is not very different from that of the control (unshocked) polyribosomes. The small (~14%) increase in new/old mRNA was also observed in another, similar experiment and could reflect a slight superiority of new mRNA in the reinitiation reactions. However, a more likely explanation for this small difference is that the pool of ^{14}C-labeled pre-mRNA was not completely depleted during the 20-min incubation with actinomycin, and that it provided a small additional source of ^{14}C mRNA during the subsequent incubation. Equilibration with such a pool would occur more rapidly in the temperature-shocked cells, in which the majority of mRNA is instantaneously converted to free mRNP.

In any event, it is clear that the ribosomes do not have a marked preference for new mRNA during the transient phase of polyribosome reassembly. This would seem to argue against any appreciable differences in initiation efficiency for new and old mRNA and, taken together with the measurements of loading ratios, would suggest that the overall translational efficiency of new and old mRNA is roughly equivalent.

This type of comparison of new and old mRNA is useful insofar as it refers to mRNAs containing poly(A) of maximum length (~180 residues) and shorter lengths (50–150 residues). It is also clear that messages such as the histone mRNAs can function with no poly(A) at all. The possibility exists, however, that the histone messages may represent a special case, and thus the question arises as to whether an mRNA that was originally polyadenylated could function efficiently if the entire poly(A) segment were removed. To answer this question we employed a cell-free protein synthesizing system and determined the relative template activities of polyadenylated mRNA and mRNA that was deadenylated by treatment with a 3′-OH specific exoribonuclease.

The protocol for experiments of this type is outlined in Figure 2. Highly purified polyadenylated mRNA, tagged with ^{3}H-uridine at a relatively low specific activity (4,350 cpm/μg) was treated with the exoribonuclease for a period of time sufficient to remove the poly(A) from more than half of the molecules, as estimated by the decrease in the proportion of mRNA binding to poly(U)-impregnated fiberglass filters. About 5% of the labeled uridine residues were rendered acid soluble as a result of the enzymatic treatment, probably because of the unavoidable asynchrony that is encountered with this processive enzyme (see Molloy et al., 1972).

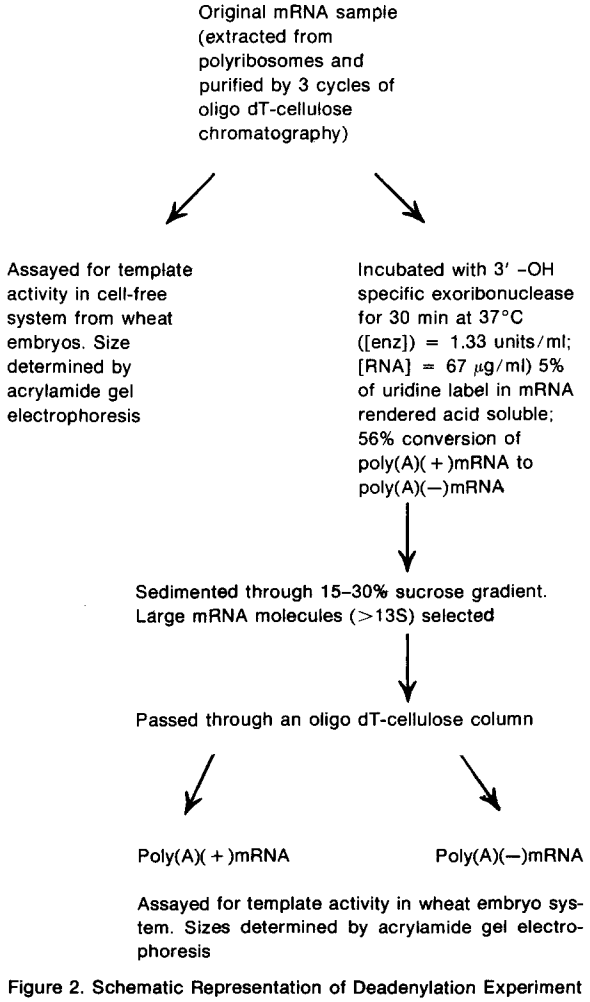

Figure 2. Schematic Representation of Deadenylation Experiment (see text)

A comparison of the size distributions of the original and digested mRNA samples, using zonal sedimentation or acrylamide gel electrophoresis, indicated a size diminution in the digested mRNA that was greater than could be accounted for by only poly(A) removal and asynchrony. The average size of the polyadenylated mRNA in the digested sample was decreased by about a factor of 2, relative to the original polyadenylated mRNA. This suggested that there was some endonuclease activity in the exoribonuclease preparation, and that the mRNA molecules suffered on the average about one break per molecule. It was further noted that the very small RNA fragments produced by the enzymatic digestion were inhibitory to the cell-free protein synthesizing system. To overcome this latter problem, the digested mRNA was sedimented through a sucrose gradient, and only the components greater than 13S selected. These large components were then separated into a deadenylated mRNA fraction and a residual polyadenylated mRNA fraction by chromatography on oligo dT-cellulose columns.

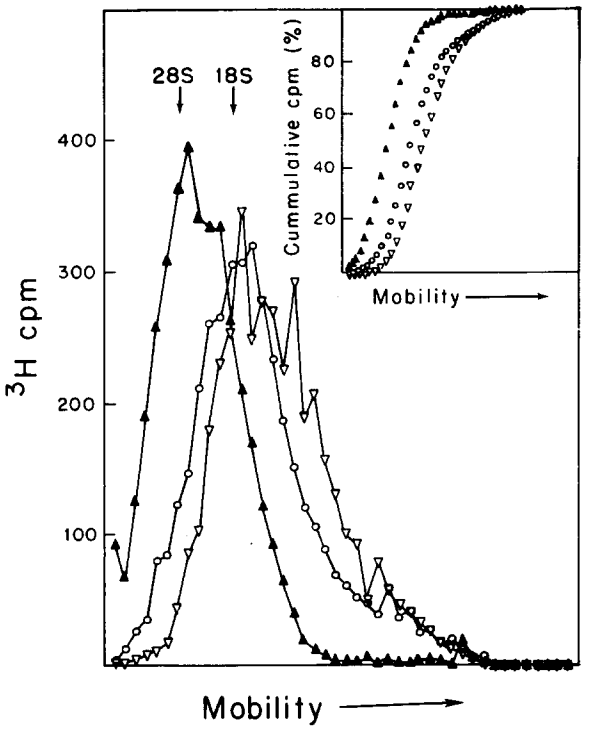

Figure 3. Analysis by Polyacrylamide Gel Electrophoresis of mRNA Preparations Used for Template Activity Assay (Table 3).

Inset shows data plotted cumulatively so that ordinate represents percentage of mRNA with size greater than corresponding abcissa value. On the basis of the semilogarithmic relationship between molecular weight and mobility (Bishop, Claybrook, and Spiegelman, 1967) and the position of the 28S and 18S ribosomal RNA markers, the modal molecular weights (50% values) of the original mRNA (▲), the poly(A)(+)-digested mRNA (○), and the poly(A)(−)-digested mRNA (▽) are estimated to be 1.2, 0.5, and 0.4 million, respectively.

The size distribution of the resultant mRNA fractions is illustrated in Figure 3, together with that of the original mRNA preparation. The residual polyadenylated and deadenylated mRNA have average molecular weights of about 0.5×10^6 and 0.4×10^6, respectively, as compared to 1.2×10^6 for the original mRNA. The difference in size between the residual polyadenylated mRNA and deadenylated mRNA reflects the greater loss of adenylate residues plus some loss of adjacent non-poly(A) residues in the latter fraction.

The comparative template activities of the three mRNA fractions, as measured in the wheat embryo protein synthesizing system, are shown in Table 3. Under the conditions of assay the incorporation was essentially proportional to the amount (in micrograms) of mRNA added, indicating that mRNA is the rate-limiting component in these measurements. The relative template activities of the digested polyadenylated [poly(A)(+)] and deadenylated [poly(A)(−)] mRNA were quite similar, with no consistent difference between them in the various experiments. Both digested mRNAs were invariably less active than the original mRNA preparation on a "per microgram" basis, presumably because some of the original mRNAs were inactivated by endonucleolytic breaks.

It is apparent from these results that an mRNA molecule that was originally polyadenylated is still capable of being translated after removal of its poly(A) segment. Although our measurements might not detect differences in the relative efficiencies for the individual steps of protein synthesis, particularly if such differences were compensating, it is striking that the overall template activities of the poly(A)(+) and poly(A)(−) mRNAs are essentially equivalent.

Discussion

In the experiments reported here we have attempted to determine whether any correlation exists between the presence of a poly(A) segment on the 3′-OH terminus of an mRNA molecule and the capacity of the mRNA to function in protein synthesis. The results of the in vivo experiments indicate that there is no apparent senescence of mRNA activity, and that old mRNA performs as well as new mRNA. Given the previously demonstrated shortening of the poly(A) segments with age (Mendecki, Lee and Brawerman, 1972; Greenberg and Perry, 1972a; Sheiness and Darnell, 1973), this would seem to indicate a lack of correlation between poly(A) length and mRNA activity.

Our in vitro experiments suggest that even the total removal of the poly(A) segments from species of mRNA that were previously polyadenylated does not noticeably affect the mRNA's ability to perform

Table 3. Comparative Template Activity of Polyadenylated and Deadenylated mRNA in a Cell-Free Protein Synthesizing System from Wheat Embryo

Input of mRNA (μg)	Incorporation of ^{14}C-leucine (cpm)		
	Original	Exonuclease digested	
	poly(A)(+)mRNA	poly(A)(+)	poly(A)(−)
	Experiment 1a		
0.5	6,290	6,970	4,670
1.0	13,270	9,910	6,440
2.0	25,600	17,140	13,210
	Experiment 1b		
0.86	5,850	2,590	3,390
	Experiment 2		
1.25	12,800	3,050	3,150

The incorporation systems in the various experiments differed in the bicarbonate concentration used in the preparation of the S23 fraction (see "Experimental Procedures"). In Experiments 1a and 1b, the mRNA preparations were those described in Figure 3, and the systems incorporated, respectively, 3,620 and 1,540 cpm of ^{14}C-leucine in the absence of added template. In Experiment 2, mRNA preparations from a different enzymatic digestion were used. The modal molecular weights, estimated as in Figure 3, were 1.4, 0.6, and 0.3 million, respectively, for the original poly(A)(+), digested poly(A)(+), and digested poly(A)(−) mRNA fractions. Endogeneous incorporation in this experiment was 450 cpm. In all experiments, endogeneous activities were subtracted from total incorporation values to give the values listed.

its translational function. Although our in vitro assay system is limited to only a few rounds of translation per message* and is thus insensitive to a possible effect of the poly(A) segments in facilitating ribosome release and/or reinitiation, we note that a natural poly(A)(-) mRNA species, such as histone mRNA, apparently has no difficulty in this regard, since it remains functionally stable throughout the DNA synthetic phase of a cell cycle (Perry and Kelley, 1973), during which it probably undergoes several thousand rounds of translation. Recently, attempts to relate poly(A) sequences to in vitro mRNA activity have been made independently in at least two other laboratories (M. Morrison and J. Lingrel, personal communication; R. Williamson, et al., 1974) with results entirely consistent with those presented here.

What, then, is the physiological role of the poly(A) sequences? Several seemingly attractive hypotheses have been investigated—for example, a possible role in transcript selection or message stabilization (see Perry, 1973) and, now, message function. All seem to be negated, at least in their simplest form, by available experimental evidence. Perhaps the elucidation of the biological significance of poly (A) will have to await a deeper understanding of the biochemical mechanisms involved in mRNA synthesis and function.

*In the experiments of Table 3, 400 p moles of ^{14}C-leucine of specific activity, 311m Ci/mmole were added. By dilution analysis, we have ascertained that the 50μl added to these incubations contribute approximately 1600 p moles of unlabeled leucine. Thus, the actual specific activity of the ^{14}C leucine is 62 m Ci/mmole which at a counting efficiency of 60% calculates to 82 cpm/pmole. Assuming complete translation, an mRNA with a coding stretch of 10^6 daltons would produce a protein of about 10^5 daltons, which on the basis of a 5% leucine content should contain about 50 leucine residues. Thus 1 μg of mRNA with an average molecular weight of the coding portion of 10^6 ($\approx$ 1 pmole) should stimulate the incorporation of 50 pmoles leucine, or 4100 cpm, for each complete round of translation.

Experimental Procedures

Methods for cultivation of mouse L cells, subcellular fractionation, RNA extraction, and mRNA purification have been detailed in previous publications (Perry and Kelley, 1968; Schochetman and Perry, 1972; Perry, Kelley, and LaTorre, 1974). Protocols for individual experiments are given in the legends of the corresponding tables and figures. Conditions for treatment of mRNA with the 3′-OH specific exoribonuclease (purified from ascites cell nuclei) have been described previously (Molloy et al., 1972). The enzyme preparation used for the present experiments appeared to contain a small amount of endonuclease contamination, which was not detectable in earlier enzyme preparations.

Assays of template activity using the wheat embryo cell-free system were performed according to Efron and Marcus (1973), except that the incubation volume was 0.20 ml, and 50 μl of wheat embryo S23 fraction were added. In the different experiments described in Table 3, varying concentrations of bicarbonate were used in preparing the S23 fraction (Marcus, Efron, and Weeks, 1973). In the range of bicarbonate sensitivity (1 to 10 mM), an increase in the bicarbonate concentration results in S23 preparations having higher values of both endogenous and template-stimulated incorporation.

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References

Adesnik, M., and Darnell, J. E. (1972). J. Mol. Biol. *67*, 397–405.

Bishop, D. H. L., Claybrook, J. R., and Spiegelman, S. (1967). J. Mol. Biol. *26*, 373–387.

Efron, D., and Marcus, A. (1973). FEBS Letters *33*, 23–27.

Greenberg, J. R. (1972). Nature *240*, 102–104.

Greenberg, J. R., and Perry, R. P. (1972*a*). Biochem. Biophys. Acta *287*, 361–366.

Greenberg, J. R., and Perry, R. P. (1972*b*). J. Mol. Biol. *72*, 91–98.

McCormick, W., and Penman, S. (1969). J. Mol. Biol. *39*, 315.

Marcus, A., Efron, D., and Weeks, D. P. (1973). In Methods in Enzymology, Part E, L. Grossman and K. Moldave, eds. (New York: Academic Press).

Mendecki, J., Lee, S.-Y., and Brawerman, G. (1972). Biochemistry *11*, 792–798.

Molloy, G. R., Sporn, M. B., Kelley, D. E., and Perry, R. P. (1972). Biochemistry *11*, 3256–3260.

Murphy, W., and Attardi, G. (1973). Proc. Nat. Acad. Sci. USA *70*, 115–119.

Nakazato, H., and Edmonds, M. (1972). J. Biol. Chem. *247*, 3365–3367.

Perry, R. P. (1973). In Molecular Cytogenetics (New York: Plenum Press), pp. 133–145.

Perry, R. P., and Kelley, D. E. (1968). J. Mol. Biol. *35*, 37–59.

Perry, R. P., and Kelley, D. E. (1973). J. Mol. Biol. *79*, 681–696.

Perry, R. P., Kelley, D. E., and LaTorre, J. (1974). J. Mol. Biol., *82*, in press.

Schochetman, G., and Perry, R. P. (1972), J. Mol. Biol. *63*, 577–590.

Sheiness, D., and Darnell, J. E. (1973). Nature New Biol. *241*, 265–268.

Sheldon, R., Jurale, C., and Kates, J. (1972). Proc. Nat. Acad. Sci. USA. *69*, 417–421.

Singer, R. H., and Penman, S. (1973). J. Mol. Biol. *78*, 321–334.

Williamson, R., Crossley, T., and Humphries, S. (1974). Biochemistry, in press.