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Short-Lived Messenger RNA in HeLa Cells and Its Impact on the Kinetics of Accumulation of Cytoplasmic Polyadenylate

[poly(A) conservation/adenine-grown cells]

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ABSTRACT **Accumulation of [^{3}H]adenine in the acid-soluble pool and in nuclear and cytoplasmic poly(A) of HeLa cells shows that the nuclear poly(A) rises along a curve similar to that of the acid-soluble pool. By use of a [^{3}H]guanosine pulse-chase experiment in adenine-grown cells, at least 35–50% of the pulse-labeled mRNA was found to have a half-life of about 1–2 hr. A mathematical model involving nuclear poly(A) synthesis and conservative transport to the cytoplasm has been derived from the new information about mRNA with a short half-life. This model predicts curves similar to those found for nuclear and cytoplasmic accumulation of poly(A). Thus, there is no necessity on kinetic grounds to invoke either nuclear turnover or cytoplasmic synthesis of poly(A).**

Both heterogeneous nuclear RNA (HnRNA) and mRNA from mammalian cells contain a 3′-terminal poly(A) segment (review in ref. 1). Previous experiments (2, 3) indicated that this material was added to HnRNA post-transcriptionally in the nucleus, after which at least 40% (perhaps more) of this poly(A) moved to the cytoplasm. This latter conclusion has been recently challenged by Perry *et al.* (4), who concluded that extensive nuclear poly(A) turnover and perhaps also cytoplasmic poly(A) synthesis must occur.

A major reason for these conclusions was the belief that most if not all mRNA in cultured cells had a long half-life (5–7), suggesting that cytoplasmic poly(A) should not be renewed by nuclear synthesis and transport nearly rapidly enough for total labeled cytoplasmic poly(A) to exceed nuclear poly(A) early in the course of [^{3}H]adenosine incorporation.

We have found that there is, in HeLa cells, a large amount of mRNA with a half-life of 1–2 hr. A kinetic model *can* be built, consonant with the experimental results in which nuclear poly(A) is the source of cytoplasmic poly(A) and all the nuclear poly(A) is transported to the cytoplasm.

METHODS AND MATERIALS

Cells and Labeling. HeLa cells were grown (doubling time, 24 hr) in suspension in Eagle's medium (8) supplemented with 0.03 mM adenine. Labeling with adenine was accomplished by concentrating cells to 2×10^5 cells per ml in the same medium; 1 μCi/ml of [^{3}H]adenine (New England Nuclear Corp., 32 mCi/μmol) was added to begin labeling. [^{3}H]Guanosine (20 mCi/μmol) was used in pulse-chase experiments by adding, for 10 min, 10 μCi/ml to cells concentrated to 4×10^6 cells per ml at 37°, followed by centrifugation at 37° and resuspension twice in Eagle's medium with 0.03 mM adenine and 0.50 mM guanosine.

Cell Fractionation and RNA Extraction. The acid-soluble fraction of cells was prepared after 5×10^6 to 10^7 cells were washed twice in conical tubes by resuspension in phosphate-buffered saline (0.15 M NaCl–0.01 M PO_4^{-3}, pH 7.4–1.5 mM $MgCl_2$) followed by addition of 0.5 ml of 0.5 M $HClO_4$. The soluble fraction was decanted and aliquots were assayed for radioactivity (50–100 μl added to Triton–toluene based scintillation mixture) and for absorbance at 260 nm. Cells were fractionated for nucleic acid isolation by the method of Penman (9). All nuclear washes, including the detergent wash, were pooled as cytoplasmic fraction.

Cytoplasmic RNA and nuclear RNA were extracted with phenol as described (3). Radioactivity in nuclear RNA labeled with [^{3}H]guanosine was assayed by digestion with RNase A (20 μg/ml) and T1 RNase(20 units/ml) in 0.01 M Tris·HCl (pH 7.4) for 60 min at 37°. 4S and 18S rRNA were assayed by gradient centrifugation. Poly(A) was isolated from RNA samples after T1 RNase digestion by affinity chromatography and measured in gel electropherograms, as the about 200-nucleotide homopolymeric unit (2, 10). Column chromatographic isolation (3×0.5-cm columns) of mRNA on poly(U)–sepharose (10) was modified by adsorbing samples in buffer containing 0.4 M NaCl (plus 0.01 M Tris·HCl, 0.01 M EDTA, and 0.2% sodium dodecyl sulfate) followed by washing at room temperature with 10 ml of the same buffer lacking NaCl, then 10 ml of the same buffer plus 10% formamide, followed by gradient elution of the attached RNA (10).

RESULTS

Soon after growing mammalian cell cultures came into use, it was shown that, among the available purine precursors, only adenine labeled the RNA of growing cells to equal specific activity with the added precursor (11, 12). In addition, only after several generations of growth in adenine was endogenous synthesis completely repressed (12). The advantage of using adenine as an RNA precursor for kinetic labeling studies then, is that the specific activity of the internal ATP pool will eventually be constant, i.e., exactly the same as the medium. The disadvantage is that the ATP pool is large and is replaced only by growth, which means that the maximum pool specific activity is not achieved for several hours. Nevertheless, it should still be possible to determine whether nuclear poly(A) accumulation continued for many hours past the plateau of

Abbreviation: HnRNA, heterogeneous nuclear RNA.

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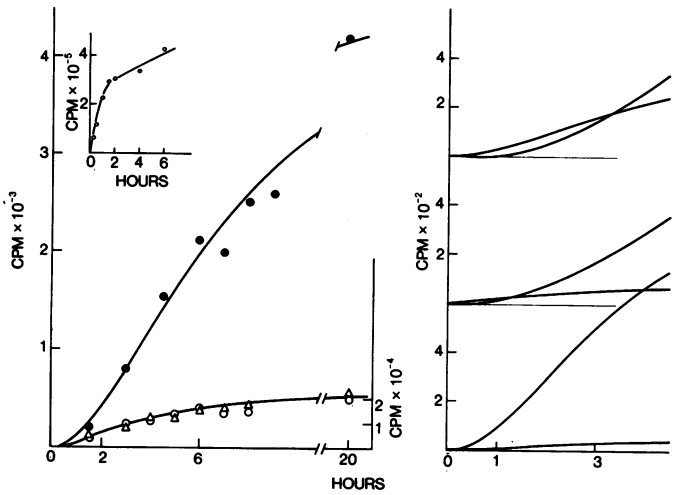

FIG. 1. The real and predicted accumulation of [^{3}H]adenine in the acid-soluble pool in nuclear and cytoplasmic poly(A). *Left panel:* Cells growing in [^{3}H]adenine (see *Methods and Materials*) accumulated radioactivity in the acid-soluble pool (Δ, right axis) and in nuclear and cytoplasmic poly(A) (left axis: ●, cytoplasmic; O, nuclear; cpm/10^7 cells is presented) as shown. The inset on the upper left side is the accumulation into the acid-soluble pool of cells of [^{3}H]adenosine (cpm/10^7 cells) when the cells were labeled according to Perry *et al.* (4). *Right panel:* Experimental data for the accumulation in HeLa cells of radioactivity into the acid-soluble pool were used to calculate the hypothetical curves of accumulation into nuclear and cytoplasmic poly(A), assuming there were three classes of mRNA with turnover times of 1, 7, and 24 hr. In all cases the cytoplasmic curve begins low and ends as the highest curve. Top, 24-hr half-life; middle, 7-hr half-life; and bottom, 1-hr half-life. The sum of the three curves is the solid line in left panel.

The model used in the derivation of the curves is:

$$k'e^{k_D t} \xrightarrow{k_0} P \xrightarrow{k_{0i}} N_i \xrightarrow{k_{1i}} C_i \rightrightarrows \begin{matrix} k_{2i} \\ k_{3i} \end{matrix}$$

where k' is the initial rate of adenine entry into the acid-soluble pool, k_D the cell doubling constant, k_{0i} the rate of nuclear poly(A) synthesis, k_{1i} the rate of nuclear-cytoplasmic poly(A) transport, and k_{2i} and k_{3i} are decay constants for poly(A) containing mRNA and poly(A) shortening, respectively; i represents any mRNA species. The details of how the computations were made for three mRNA species, and how changes of various parameters (amount of mRNA in any given species, the turnover times, etc.) affect the curves will be published separately (L. Puckett and J. E. Darnell, in preparation).

specific activity of the acid-soluble pool. Fig. 1 shows that when adenine-grown cells were exposed to [^{3}H]adenine, the acid-soluble radioactivity rose for many hours, and the nuclear poly(A) content rose along a very similar curve, i.e., labeled nuclear poly(A) accumulation continued only so long as the acid-soluble pool rose. [The acid-soluble pool measurement made in our experiments is simply total acid-soluble cpm per 10^7 cells, since it has been shown that adenine nucleotides, mainly ATP, account for over 60% of the total acid-soluble nucleotides (13, 14).]

The radioactivity in the acid-soluble pool of L cells labeled with [^{3}H]adenosine according to the protocol of Perry *et al.* (4) also continued to rise at least for 8 hr. Such a rise might also account for a continuous increase in labeled nuclear poly(A). Thus, conclusions about nuclear poly(A) turnover based on a continuing nuclear accumulation of poly(A) seem unjustified.

TABLE 1. *Distribution of labeled poly(A) after a brief pulse of [^{3}H]adenine*

Labeling time (min)	Cytoplasm (cpm)	Nucleus (cpm)
2	Undetectable, undetectable	150, 155
5	140, 80	220, 230

Cells grown in adenine-containing medium (0.03 mM) were concentrated to 4 × 10^6 cells in the same medium and labeled by addition of 2 mCi of [^{3}H]adenine for 2 or 5 min. The labeled nuclear and cytoplasmic poly(A) were then purified and analyzed by gel electrophoresis. The maximum cpm per gel slice in 2-min nuclear samples was 85 cpm above background. The comparable gel slice in the cytoplasmic sample was equal to machine background.

The next consideration was whether the rate of nuclear poly(A) accumulation could account for the fast appearance of cytoplasmic poly(A) when the half-life of mRNA appeared to be so long. Table 1 shows that with [^{3}H]adenine (total concentration 0.03 mM) as the labeled precursor to poly(A), the nucleus contained more labeled poly(A) than the cytoplasm after very short labeling times, just as had first been found with a pulse of [^{3}H]adenosine of high specific activity (3). This analysis for poly(A) involved identification by gel electrophoresis of "7–9S" ribonuclease-resistant, chromatographically separated species (1, 2). This is especially important for cytoplasmic samples after short labeling times because short mitochondrial poly(A) may make a substantial contribution to briefly labeled total cytoplasmic poly(A) (15). During the course of longer exposures to [^{3}H]adenine, and as was previously seen with [^{3}H]adenosine (2, 3), the labeled cytoplasmic poly(A) soon exceeds the labeled nuclear poly(A) and the two continue to rise as the specific activity of the pool rises (Fig. 1). This rapid cytoplasmic rise required further exploration in light of three points: (*i*) at steady state the nuclear poly(A) makes up about 1/10 to 1/20 of the total cellular poly(A) (16, 17); (*ii*) a large proportion of mRNA is believed to exit to the cytoplasm only after a 20- to 30-min delay (4, 18); and (*iii*) a majority of the cell mRNA was thought to have a long half-life (13–17). Together these results led Perry *et al.* (4) to suggest cytoplasmic poly(A) synthesis and nuclear poly(A) turnover.

A number of the recent measurements of mRNA half-life have relied on uridine labeling followed by removal of label and measurements of the decline of labeled mRNA beginning 4–5 hr later (5, 7). Since total labeled mRNA increases for some hours after the withdrawal of [^{3}H]uridine, these experiments would underestimate the amount of rapidly turning-over mRNA because such mRNA would be proportionately diminished by the time experimental observations began. In adenine-grown cells, a much more effective chase might be expected after a [^{3}H]guanosine pulse. The GTP pool is the smallest triphosphate pool in HeLa cells (13, 14), and in adenine-grown cells the labeling of the ATP pool by guanosine should be minimal. Accordingly, cells were labeled with a 10-min exposure to guanosine, the label was removed by centrifuging the cells, and unlabeled guanosine was added when the cells were suspended. The total acid-precipitable radioactivity in the culture was maximal within about 2 hr, and only GMP

(>98%) was labeled in HnRNA, as determined by enzymatic hydrolysis and electrophoretic separation of nucleotides (19).

As an assay of whether a chase had been effected, the total radioactivity in tRNA (whose precursor pool is small) (20) was examined and found to be virtually constant within about 3 hr after label and chase (Fig. 2). Moreover, the total radioactivity in the nucleus began to decline sharply after 120 min, indicating a much more effective chase than has been observed with uridine (21). 18S rRNA continued to accumulate somewhat longer than 4S RNA, as would be expected because of the pool of labeled nucleolar ribosomal pre-RNA.

Labeled mRNA was then assayed in cells pulse-chased with guanosine. A technique similar to that used by others was used for mRNA isolation (22). Total cytoplasmic poly(A)-terminated RNA was selected by poly(U)–sepharose binding and elution; no detectable labeled rRNA contaminated the bound fraction and the sedimentation size of the bound fraction was similar to [^{3}H]uridine-labeled mRNA (5–7). Both 2 hr and 5 hr after the exposure to [^{3}H]guanosine, 60–65% of the labeled poly(A)-terminated cytoplasmic molecules were in polyribosomes, and 35–40% in more slowly sedimenting structures. A similar distribution among cytoplasmic structures had been found earlier for the total cytoplasmic poly(A) (3). Thus, the total [^{3}H]guanosine-labeled poly(A)-terminated cytoplasmic fraction, which has the appropriate sedimentation profile for mRNA and is mostly associated with polyribosomes, certainly includes the labeled mRNA. Whether every poly(A)-terminated cytoplasmic molecule serves as mRNA is unknown. However, we were concerned mainly in this study with entry and turnover of the total poly(A)-containing cytoplasmic fraction, and this fraction will be referred to as mRNA. Loss of radioactive mRNA from the chromatographically bound fraction has been used as a measure of mRNA turnover. This loss could theoretically be due to loss of poly(A), but not to loss of mRNA. This limitation applies to our experiments as it does to earlier work. The chromatographic procedure that was used in the present experiments causes retention on the poly(U)–sepharose of mRNA with shortened poly(A) (5) as well as the long 200-nucleotide poly(A). Thus, when we find a decrease in bound radioactive mRNA, we are not observing simply a failure to bind based on poly(A) shortening alone.

Fig. 3 shows the decay curve of total radioactivity in mRNA as well as radioactivity in mRNA compared to the radioactivity in the 18S rRNA in cells pulse-chased with [^{3}H]guanosine. The total radioactivity in mRNA begins to decline after 3 hr, and between 3 and 6 hr there is a 50% loss of total labeled mRNA with a 30% loss between 3 and 4 hr. Perhaps the best means of monitoring the stability of mRNA (13, 14) during the chase is to compare radioactivity in mRNA to the radioactivity in 18S rRNA, which is stable in the cytoplasm and has about the same lag in the nucleus (20–30 min) (16, 17, 23) before cytoplasmic appearance. Thus, during the period of decline in total nuclear radioactivity when some newly labeled molecules are still appearing, the ratio of labeled mRNA to 18S rRNA serves as a sensitive measure of the loss of rapidly turning over mRNA. Compared to radioactivity in 18S rRNA, the relative amount of labeled mRNA began to decline within 2 hr and about 50% was lost between 2 and 3 hr of chase. The remaining labeled mRNA decays more slowly, comparable to that seen in the experiments of Singer and Penman (5). Thus, there is clear evidence for a substantial amount (35–55%) of newly appearing, guanosine-labeled mRNA which disappears

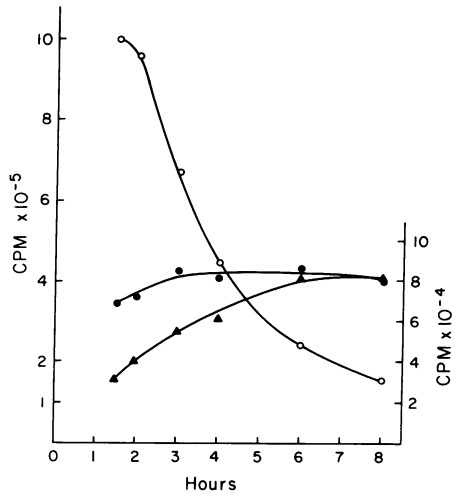

FIG. 2. Pulse-chase of HeLa cell RNA with [^{3}H]guanosine. Cells grown for many generations in adenine (0.03 mM) were pulse-labeled (see *Methods and Materials*) with [^{3}H]guanosine for 10 min (0 time). Cells were washed and resuspended for analysis of radioactive RNA at indicated times. Radioactivity in total nuclear RNA (○, left axis); 4S RNA (●, right axis); and 18S rRNA (▲, right axis) are presented in cpm/3 × 10^7 cells.

with a half-life as fast as or faster than (1–2 hr). Accurate estimates are not possible of the exact turnover time or what fraction of an instantaneous label would be found in mRNA classes with various half-lives, since the [^{3}H]guanosine chase was not perfect; labeled nuclear RNA only began to decline 2 hr after the introduction of label, and new 18S continued to appear until 4 hr. A continuing influx of label decreases the chance of observing rapidly turning over mRNA, yet we still see about 50% with a short half-life. Clearly a considerable fraction of the newly appearing cytoplasmic HeLa cell mRNA could have a very rapid half-life. This does not mean that at steady state a similar fraction of the *total* mRNA molecules in the cell are short-lived.

The cell fractionation procedure used in these experiments described thus far would not have separated mitochondrial mRNA from total cytoplasmic mRNA, although mitochondrial RNA should represent only a small proportion of the total labeled cytoplasmic RNA (15). To rule out the possibility that the short-lived fraction was mitochondrial in origin, a [^{3}H]guanosine chase experiment was performed using only the extra-mitochondrial cytoplasmic fraction. The ratio of mRNA to 18S rRNA during the chase again indicated a turnover time of 1 hr or less for at least 40% of the mRNA.

A MODEL FOR THE KINETICS OF LABELING OF CELLULAR RNAS AND DISCUSSION

Mathematical analysis of the accumulation of nuclear and cytoplasmic poly(A) with a view toward determining whether a simple precursor-product relationship might exist between nuclear and cytoplasmic poly(A) species has been reported (4). The equations used in these studies included a single transport time from nucleus to cytoplasm and a single turnover time for the mRNA of 600 min. The data for poly(A) accumulation in either the nucleus or cytoplasm did not fit such a model, and the conclusions were drawn that either nuclear turnover or cytoplasmic poly(A) synthesis or both must occur (4). With the finding that mRNA does not all

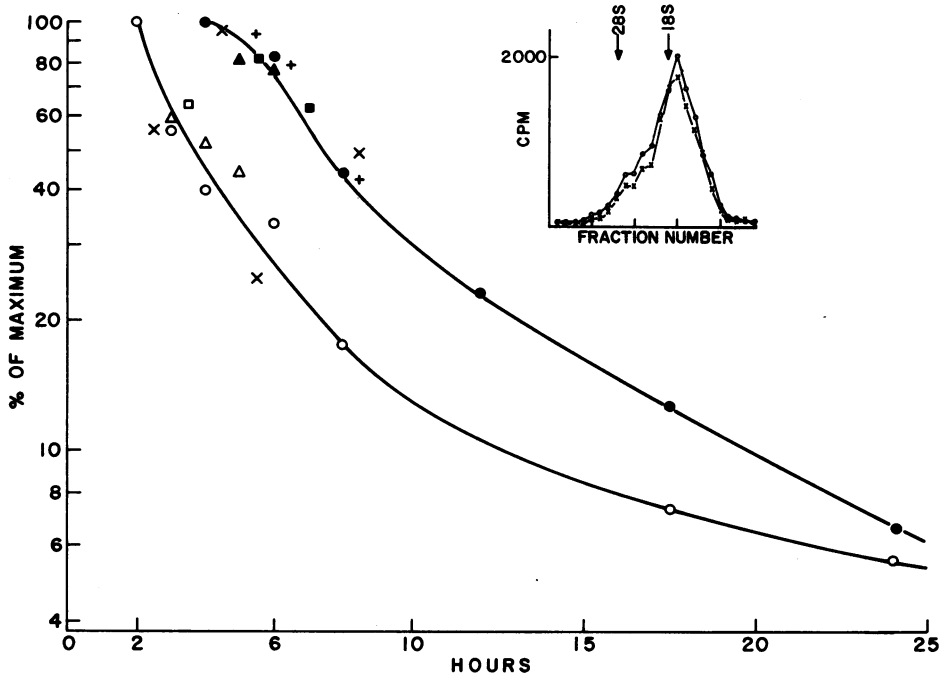

FIG. 3. Turnover of [^{3}H]guanosine-labeled mRNA. Cells pulse-labeled with [^{3}H]guanosine and chased in unlabeled medium (see legend of Fig. 2 and *Methods and Materials*) were assayed for radioactivity in cytoplasmic poly(A)-terminated molecules at indicated time intervals. Different symbols represent different experiments. Insert shows zonal sedimentation profile of poly(U)–sepharose-bound fraction and lack of rRNA contamination (O, 2-hr; ×, 5-hr samples; same number of cpm was placed on the gradient for analysis). Open symbols, cpm in mRNA compared to cpm in 18S rRNA, with the 2-hr sample considered as 100%. Closed symbols, cpm in mRNA/4 × 10^7 cells, with the 4-hr sample considered as 100%.

turn over with a long half-life, we have described a more complicated model for poly(A) accumulation involving three mRNA components of different half-lives, one of which is quite fast (Puckett and Darnell, in preparation). The model utilizes the data from cells labeled with [^{3}H]adenine. The equations used in the model are based on the original equations of Roberts *et al.* (24), and they describe the flow of radioactivity from medium → ATP pool → nuclear poly(A) → cytoplasmic poly(A). The experimental measurements possible are radioactivity in the acid-soluble pool and in nuclear poly(A) and cytoplasmic poly(A). The model takes the data for increase in acid-soluble pool and predicts the curves of increase for nuclear poly(A), which is taken to be the sole precursor to cytoplasmic poly(A), based on the amounts of three different turnover classes of mRNA.

The three RNA species considered in the model were the two long-lived mRNA species (7 and 24 hr) described by Singer and Penman (5) and a rapid turnover species with a half-life of 1 hr as indicated by this work. The proportions of the steady-state amount of poly(A) contributed by each of these classes was 15, 27, and 58% for the species with turnover times of 1, 7, and 24 hr, respectively. In the approximation of the amounts of poly(A) in each species it was assumed that the previously observed poly(A) shortening (16, 17) was the same (half-time of 6 hr) for each class of mRNA. Fig. 1 (right panel) shows diagrammatically the three idealized precursor-product curves for the three chosen mRNA species. The rapidly turning-over mRNA has a big impact on the early course of cytoplasmic poly(A) accumulation, while the slowly turning-over class is responsible for a slow rise in the nuclear poly(A). When summed together, the three curves give an accumulation curve of nuclear and cytoplasmic poly(A) not dissimilar to the experimental data. The solid lines on the left side of Fig. 1 are predicted lines plotted together with the actual data.

The apparent similarity of actual and predicted curves does not by any means prove the model to be correct. However, it clearly points up the hazards of attempting to prove turnover of nuclear poly(A) or invocation of cytoplasmic poly(A) synthesis by kinetic arguments. Without very firm knowledge of the details of stability of the cytoplasmic fractions in a multistep situation such as: medium precursor → pool ATP → nuclear poly(A) → cytoplasmic poly(A) → acid-soluble (? AMP)—it is only possible to suggest the relationship of one cellular component to another through a kinetic approach.

In summary, we return to consider the main points at issue concerning poly(A) metabolism in cultured mammalian cells. When labeling times are restricted to less than 5 min, the vast majority of radioactivity in the 200-nucleotide unit of poly(A) is in the nucleus (Table 1 and ref. 10). Unless there is a special cytoplasmic ATP pool that labels more slowly than the nuclear pool this experiment alone identifies the nucleus as the site of synthesis of the 200-nucleotide poly(A) unit in cultured mammalian cells. It has been shown (25) that poliovirus RNA is constructed in the cytoplasm of HeLa cells from an acid-soluble pyrimidine pool that is in ready equilibrium with the pool from which nuclear RNA is made. Thus, evidence against special slowly equilibrating pools exists, at least for pyrimidines. In addition, the kinetic considerations from the present work indicate the possibility of only nuclear synthesis of the 200-nucleotide poly(A) unit.

Even if it is true then, that poly(A) synthesis in cultured (somatic) cells is nuclear, this, of course, does not mean that all poly(A) in all mRNA molecules arises in the nucleus. In

several situations, different from somatic cell mRNA biogenesis, cytoplasmic poly(A) synthesis occurs. Vaccinia virions, for example, were shown very early to be the site of synthesis of poly(A) attached to vaccinia mRNA; this virus replicates in the cytoplasm (26). Mitochondria synthesize poly(A), albeit a smaller unit, attached to mitochondrial mRNA (26). Fertilized sea urchin eggs, even when enucleated, synthesize poly(A) which is attached to larger RNA (27, 28). In this last case it is not clear what type of molecule is the immediate receptor for poly(A), although it is clear that the material becomes mRNA soon after poly(A) addition. Fertilized eggs might be a case of truly "cytoplasmic" enzymes adding poly(A) to mRNA or they might represent the transport of the whole mRNA processing apparatus ("stored HnRNA") so that events are carried out in the cytoplasm which in somatic cells normally occur in the nucleus. Eggs do contain 1000 times as much cytoplasm relative to nuclear material as do somatic cells (37), and certain eggs perform functions that are clearly "nuclear," e.g., virus DNA replication (29).

In addition to the question of site of poly(A) synthesis, the problem of the extent of transport of nuclear poly(A) to cytoplasm is controversial. With the knowledge that rapidly turning over mRNA exists, a model involving nuclear synthesis and quantitative transport can be described that fits the data for labeled poly(A) accumulation. A detailed proof of whether some nuclear poly(A) turnover occurs, we suspect, will come not from further kinetic arguments, but from experiments examining the nuclear and cytoplasmic sequences to which poly(A) is attached.

A final point about the present experiments does not concern the metabolism of poly(A). The finding of rapidly turning-over mRNA opens the chance to further divide and thus understand the multitude of types of mRNA molecules found in the cytoplasm of complicated cells. For example, mRNA molecules from repeated sites in DNA have recently been described (30). Is there a correlation between rate of turnover and type of DNA from which the mRNA originates? Many physiologic experiments have implicated short-lived cytoplasmic factors in translational control of protein synthesis (31). Are these factors specified by the short-lived mRNA observed here? Clearly a technique for observing short-lived mRNA molecules should prove useful.

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