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Stability of Cytoplasmic Messenger RNA in HeLa Cells

(actinomycin D/benzoylated-DEAE–cellulose/poly(T)–cellulose/nucleoside triphosphate pools/cold chase)

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ABSTRACT **The metabolic stability of pulse-labeled or long-term labeled messenger RNA (mRNA) from cytoplasmic free polysomes was measured in HeLa cells, with chase conditions that do not involve inhibitors of RNA synthesis, and chromatography on benzoylated-DEAE–cellulose or poly(T)–cellulose for isolation of mRNA. For these studies, a new chase technique was developed that allows analysis of the stability of mRNA labeled during a short [^{3}H]uridine pulse. Pulse-labeled and long-term labeled mRNA were found to decay with an estimated average half-life of about 2 and 3 days, respectively, much longer than hitherto assumed.**

Because of the lack of an effective cold-chase technique and of methods for purification of messenger RNA (mRNA), the main approach used for the study of metabolic stability of cytoplasmic mRNA in animal cells has been the analysis of the quantitative behavior and synthetic capacity of cytoplasmic polysomes after further mRNA synthesis has been arrested by actinomycin D. The results of these experiments have suggested that, while in differentiated cell types such as erythrocytes (1, 2) or liver cells (3) the bulk of the mRNA is considerably stable, the half-life of cytoplasmic mRNA in rapidly growing cells is relatively short, 3–4 hr (4, 5). The basic premise upon which these experiments rely is that polysome breakdown is a direct measurement of normal degradation of pre-existing mRNA. Recent studies on the secondary toxic effects of actinomycin D raise serious doubts as to the validity of this assumption (6–8).

Because most of these toxic effects of the drug would lead to an underestimate of the stability of mRNA in rapidly growing cells, we felt it to be essential that this question be again examined under physiological conditions. In the present work, we report experiments by which we have measured the half-life of mRNA of cytoplasmic free polysomes in HeLa cells, using chase conditions that do not involve inhibitors of RNA synthesis, and chromatography on benzoylated-DEAE–cellulose or poly(T)–cellulose for isolation of mRNA. We found that the stability of the bulk of the free polysome mRNA population is much greater than previously reported, with an estimated average half-life of about 3 days.

MATERIALS AND METHODS

Cells and Method of Growth. HeLa cells were grown in suspension in modified Eagle's medium (9) with 5% calf serum.

Labeling Conditions. For short-term labeling of RNA or DNA, HeLa cells (1 to 2 × 10^5/ml) were exposed to [*5*-^{3}H]-uridine (20–30 Ci/mmol; 0.1 μCi/ml in the kinetic experiments, 2.5–5.0 μCi/ml in the experiments for RNA analysis), or to [*8*-^{3}H]adenosine (28 Ci/mmol, 1.0 μCi/ml), or to [*methyl*-^{3}H]thymidine (29 Ci/mmol, 1 μCi/ml), for the times indicated.

Long-term labeling of mRNA for stability measurements was performed by growth of the cells (0.5 to 1.0 × 10^5/ml) in the presence of 2.5 μCi/ml of [*5*-^{3}H]uridine (20–30 Ci/mmol) for 24 hr. In order to ensure, as much as possible, a uniform incorporation of the precursor into the mRNA over the entire 24-hr period, additional label was administered at 8, 16, and 22 hr of incubation to bring the amount of radioactivity in the medium to its original level (10). As an internal control to monitor the recovery of mRNA from various samples in the analysis of mRNA stability, a constant amount of cells grown for 1 or 2 generations in the presence of 0.03 μCi/ml of [*2*-^{14}C]uridine (62 Ci/mol), with an additional 0.03 μCi/ml of label being administered 2–3 hr before harvesting, was added to the sample for each time point.

For measurement of the rate of protein synthesis, HeLa cells, suspended at 1 × 10^5/ml in modified Eagle's medium with 0.2 mM leucine and 5% dialyzed serum, were incubated with 0.2 μCi/ml of [^{3}H]leucine (40 Ci/mmol). Total acid-precipitable, alkali-resistant radioactivity incorporated was determined (11).

For analysis of labeling of nucleotide pools, HeLa cells, suspended at 1 × 10^5/ml in modified Eagle's medium with 1 mM phosphate and 5% dialyzed serum, were grown in the presence of carrier-free [^{32}P]orthophosphate (1 μCi/ml) for 12–24 hr. By this time, the nucleoside triphosphate pools are uniformly labeled with ^{32}P (12). After ^{32}P labeling, [*5*-^{3}H]uridine (2.5–5.0 μCi/ml), or [*methyl*-^{3}H]thymidine (1 μCi/ml), or [*8*-^{3}H]adenosine (1.0 μCi/ml) was added to the cultures for the times indicated.

Chase Conditions. "Cold" Chase: After cells were labeled for 30 min or 24 hr with [*5*-^{3}H]uridine, unlabeled 10 mM uridine and 5 mM cytidine were added. The culture was then cooled in an ice bath to 4° (in a 12-min period), allowed to remain at this temperature for 3 hr under gentle stirring, warmed in a water bath to 37° (in 12 min), and kept thereafter at this temperature. Warm Chase: 10 mM unlabeled uridine (in some experiments, also 5 mM unlabeled cytidine) were added to the growth medium, or the cells were centrifuged and suspended in fresh medium, as specified below.

Abbreviations: SDS, sodium dodecyl sulfate; BDC, benzoylated-DEAE–cellulose.

Free Polysome Isolation. A HeLa-cell extract was prepared (13), with 1.5 mM $MgCl_2$ in the homogenization medium. Free polysomes were isolated from the postmitochondrial supernatant by centrifugation through a discontinuous sucrose gradient (14). The polysome pellet was suspended in 1.0 ml TKM [50 mM Tris buffer, pH 6.7 (25°)-25 mM KCl-2.5 mM $MgCl_2$], layered on a 15-30% sucrose gradient in TKM, and centrifuged for 90 min at 25,000 rpm at 2° in an SW25.1 Spinco rotor. For analysis of EDTA sensitivity of the polysomes, the polysome pellet was dissolved in TKV (TKM, with 10 mM EDTA instead of $MgCl_2$) and centrifuged, as described above, through a 15-30% sucrose gradient in TKV.

RNA Extraction and Analysis. RNA was extracted from polysomes by the sodium dodecylsulfate (SDS)-Pronase-phenol method (15). For sedimentation analysis, the RNA was centrifuged for 15 hr at 26,000 rpm at 20° in an SW25.3 Spinco rotor through a 15-30% sucrose gradient (prepared over a 1-ml cushion of 64% sucrose) in SDS buffer.

Benzoylated-DEAE-Cellulose (BDC) Chromatography. Polysomal RNA was analyzed on BDC columns (16). The resin was either prepared in this laboratory or purchased from Schwarz/Mann or Boehringer-Mannheim Corp.

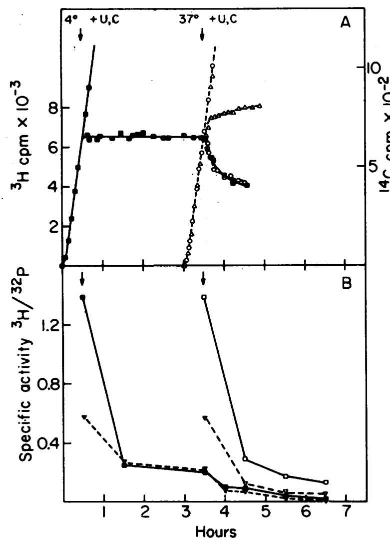

FIG. 1. (*A*) Kinetics of net incorporation of [^{3}H]uridine by HeLa cells and decay of labeled RNA by three different pulse-chase techniques. Three HeLa cell cultures were labeled for 30 min with [^{3}H]uridine; one was cooled for 3 hr in the presence of 10 mM uridine and 5 mM cytidine, and then warmed to 37° (■——■), the other two were chased at 37° by addition of 10 mM uridine (△——△), or 5 μg/ml of actinomycin D (○——○). The data plotted are the acid-precipitable cpm in aliquots of the cultures after the cells were washed with a salt solution. The labeling data for the latter two cultures are displaced on the axis of abscissae, so as to compare the effectiveness of the chase at 37° with that of the cold-treated culture. The actinomycin experimental data are redrawn from Houssais and Attardi (20). (*B*) Specific activities of the UTP and CTP acid-soluble pools in cells labeled for 30 min with [^{3}H]uridine and either subjected to a "cold" chase and then warmed, or directly chased at 37° with 10 mM uridine and 5 mM cytidine. The cells had been previously labeled for 24 hr with [^{32}P]orthophosphate, and the specific activity data are expressed as ^{3}H to ^{32}P ratios (11). "Cold" chase: ■——■, UTP; ▼——▼, CTP. Warm chase: □——□, UTP; ▽——▽, CTP.

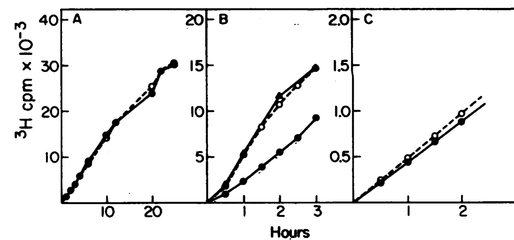

FIG. 2. Kinetics of net protein (*A*), RNA (*B*), or DNA synthesis (*C*) in cells subjected to "cold" chase. The time-courses of synthesis measured in "cold" chased cultures immediately after the cooling period, ●——● or, for RNA, also after 3 hr rewarming ▲——▲, are compared to those of untreated cultures at the same cell concentration ○——○. The incorporation of [8-^{3}H]adenosine into RNA was measured as acid-precipitable cpm solubilized by treatment with 0.5 N NaOH at 30° for 22 hr. The [8-^{3}H]-adenosine- and [^{3}H]thymidine-labeling data are corrected for differences in specific activity of the ATP and TTP pools, respectively.

Poly(T)-Cellulose Chromatography was performed according to Kates *et al.* (17), except that RNA was applied to the column in 0.12 M NaCl-10 mM Tris, pH 7.4-1 mM EDTA, and the column was washed with the same buffer.

Nucleotide Pool Analysis. The nucleoside triphosphates were isolated from cells long-term labeled with [^{32}P]orthophosphate, and labeled for different times with tritiated precursors, by two-dimensional thin-layer chromatography on PEI-cellulose, as described (11).

RESULTS

Effectiveness and physiological effects of the "cold" chase technique

The conventional pulse-chase technique, involving the use of a large excess of unlabeled precursor to dilute the intracellular pools, is ineffective in the study of RNA metabolism in animal cells because of the continued RNA labeling from the large triphosphate pools, which are fed by the turnover of unstable RNA species. In view of the need for an effective chase technique in the present work, an effort was made to develop chase conditions that would overcome this difficulty.

Since at least some of the enzymes involved in nucleotide phosphorylation may remain active at reduced temperatures (18, 19), a chase procedure was devised in which addition of an excess of unlabeled precursors was coupled with a relatively brief cold treatment, in an attempt to wash out the triphosphate pools while RNA synthesis and degradation are arrested*. A comparison of this "cold" chase with a warm chase using an equal concentration of exogenous unlabeled uridine is shown in Fig. 1*A*. After a 30-min [^{3}H]uridine pulse, the warm chase fails to stop further incorporation of label into RNA, which continues for at least 2 hr. By contrast, after the "cold" chase, there is an immediate decay of the incorporated label, with a reduction of about 35% during a 40-min period after the culture is rewarmed. The initial kinetics of decline in acid-precipitable counts per min (cpm) is similar to that found after further RNA synthesis is blocked with 5 μg/ml of actinomycin D; this decay represents in most part the turnover of heterogeneous nuclear RNA (20), which

* A detailed description of this procedure will be published elsewhere.

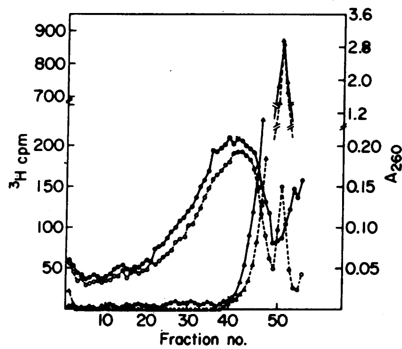

FIG. 3. Sedimentation patterns in sucrose gradients of free polysomes from cells labeled with [^{3}H]uridine for 20 min before and after disruption with EDTA. The free polysome pellet from 1.6×10^{8} cells was divided into two equal parts: one half was analyzed in sucrose gradient in TKM, the other half was analyzed in sucrose gradient in TKV. ○- - -○, A_{260} TKM; △- - -△, A_{260} TKV; ●——●, ^{3}H cpm TKM; ▲——▲, ^{3}H cpm TKV.

in the warm chase is masked by a continued high rate of incorporation. The basis for the effectiveness of the "cold" chase procedure is shown in Fig. 1*B*. During the 3-hr "cold" treatment, there is no change in the amount of incorporated radioactivity, while the specific activities of the UTP and CTP pools are reduced to 13 and 38%, respectively, of their original values. This reduction in specific activity is the result of two cooperative effects: the increase by a factor of about 2.5 in the UTP pool size, due to the high uridine concentration in the medium†, and a loss of 70% of the total acid-soluble radioactivity from the cells.

In Fig. 2, the kinetics of net synthesis of RNA, DNA, and protein after the "cold" chase are compared to control kinetics. The net incorporation of [^{3}H]leucine into protein starts immediately after rewarming (within 2 min from transfer of the culture to the 37° water bath), and proceeds at a rate identical to that of the control culture, when analyzed both within the first few minutes after rewarming (not shown) and over an extended period (Fig. 2). These observations suggest that not only does the "cold" chase not impair polysome protein synthesis, but also it allows the arrival of mRNA at the polysomes to occur at a normal rate. Similarly, the measured rate of DNA synthesis is about 90% that of control, the difference being presumably not significant. This result, coupled with an observed steady exponential increase in cell number and a normal generation time (about 24 hr), indicates that no significant degree of synchronization was induced by the cold shock. There does exist, however, a transitory inhibition of about 35% of the rate of net RNA synthesis in the cell. This inhibition, which is caused by the high concentration of exogenous uridine, gradually disappears until, by 3 hr, the rate of net RNA synthesis returns to normal.

Isolation of free polysome mRNA

Since benzoylated-DEAE-cellulose (BDC) chromatography has been successfully used in purification of bacterial mRNA (16), isolation of HeLa cell mRNA was attempted by such a procedure. Free polysomes from cells pulse-labeled for 20 min with [^{3}H]uridine and uniformly labeled with [^{14}C]uridine were isolated. These polysomes are more than 95% pure, as judged by the sensitivity to EDTA of both the UV absorbing and the radioactive material (21) (Fig. 3). Fig. 4*A* shows the sedimentation profile of polysomal RNA. Clear [^{14}C]-uridine peaks corresponding to ribosomal RNA (rRNA) and transfer RNA (tRNA) are seen, while the pulse-labeled mRNA shows a heterogeneous sedimentation profile extending from about 8 S to more than 40 S, with a broad peak centered around 20 S. The small peak of ^{3}H label found at the bottom of the tube is RNA that has been prevented from pelleting by the cushion of 64% sucrose.

The portion of the gradient indicated by arrows in Fig. 4*A* was pooled and chromatographed on BDC at pH 3.5. Fig. 4*B* shows the RNA eluted, as a single peak, with 1 M NH_4Cl in urea buffer (8 M urea-0.1 M acetic acid, pH 3.5); this RNA includes about 90% of the ^{3}H-labeled mRNA (in different experiments, the yield of mRNA varied between 70 and 95%) and only about 5% of the ^{14}C-labeled RNA. The sedimentation profile of the eluted pulse-labeled material is identical to that shown in the original gradient (Fig. 4*C*), showing that there was no degradation or preferential isolation of any size class of mRNA. The ^{14}C curve shows a small peak at 18 S and a smaller one at 28 S; these peaks presumably represent a small amount of eluted rRNA, corresponding to about 2% of that originally present in the preparation.

The basis of the separation on BDC at pH 3.5 of mRNA and rRNA is not clear (16). Thus, although this procedure appeared to be effective for isolation of HeLa cell mRNA, we felt it to be desirable to check the results with an independent technique. For this purpose, the procedure for isolation of mRNA from mammalian and other eukaryotic polysomes on poly(U)- or poly(T)-cellulose columns (22, 23) [exploiting the existence of poly(A) stretches covalently linked to the mRNA molecules (22-24)] was used. The sedi-

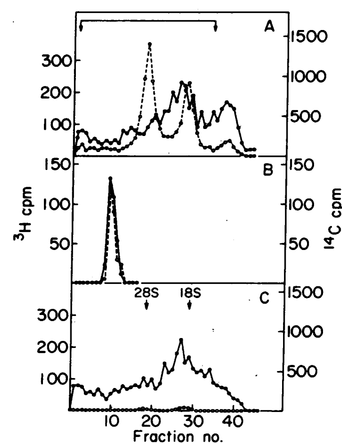

FIG. 4. Sedimentation patterns in sucrose gradients of HeLa cell polysomal RNA before and after BDC chromatography. (*A*) Sedimentation profile of RNA extracted from a mixture of cells labeled for 20 min with [^{3}H]uridine and cells labeled for 48 hr with [^{14}C]uridine and chased for 24 hr with 1 mM uridine. (*B*) The portion of the gradient indicated by *arrows* in (*A*) was collected by ethanol precipitation and centrifugation, and chromatographed on BDC, pH 3.5. The RNA eluted with 1 M NH_4Cl in urea buffer is shown. (*C*) A portion of the RNA eluted from the BDC column was run in sucrose gradient as in (*A*). ●——●, ^{3}H cpm; ○- - -○, ^{14}C cpm.

† No such effect was observed for the CTP pool (its size was about one-tenth of that of UTP after the chase), while the ATP pool was reduced to one half.

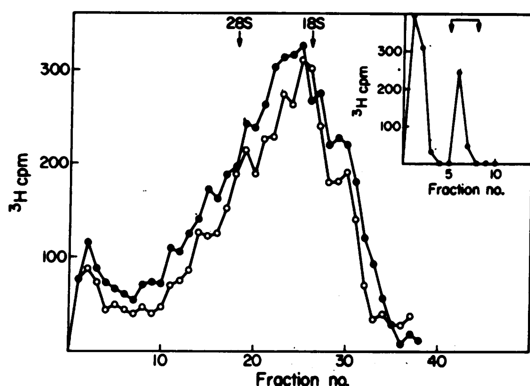

FIG. 5. Comparison of sedimentation patterns of pulse-labeled polysomal RNA isolated by BDC or poly(T)-cellulose chromatography. A portion of polysomal RNA from cells labeled with [^{3}H]uridine for 30 min was run on a poly(T)-cellulose column (*insert*), and an equal portion on a BDC column. The material eluted with 10 mM Tris, pH 7.4–1 mM EDTA from the poly(T)-cellulose column (*arrows* in *insert*) and that eluted from the BDC column were collected by ethanol precipitation and centrifugation, and run on sucrose gradients, as in Fig. 4. ●——●, poly(T)-cellulose-isolated RNA; ○——○, BDC-isolated RNA.

mentation patterns of mRNA pulse-labeled for 30 min isolated on a BDC and a poly(T)-cellulose column (insert) are compared in Fig. 5. The ^{3}H profiles of the mRNA are substantially identical, with the recovery of the ^{3}H label being about 15% higher with the poly(T)-cellulose column. A similar sedimentation pattern of HeLa mRNA separated on a poly(T)-cellulose column has been recently published (25). In the present work, both BDC and poly(T)-cellulose chromatography were used to isolate mRNA for analysis of its metabolic stability.

Metabolic stability of free polysome mRNA

By the techniques described above, the metabolic stability of free polysome mRNA was investigated. In order to be able to follow the fate of both the faster turning over and the more stable components of mRNA, the cells were labeled either for a short time (30 min) or for 24 hr.

Fig. 6 summarizes the results of several experiments measuring the rate of decay, after "cold" chase, of free polysome mRNA labeled for 30 min or for 24 hr with [^{3}H]uridine, and isolated by either BDC or poly(T)-cellulose chromatography. The zero-time point in these experiments was measured immediately after the temperature of the rewarmed culture reached 37°. In the experiments involving a 30-min pulse, this zero-time value was corrected (see legend of the figure) both for the arrival at the polysomes of mRNA labeled during the pulse (insert) and for continued incorporation of the label remaining in the nucleotide pools. No such correction was applied to the 24-hr labeled mRNA, because of its small value. mRNA labeled with [^{3}H]uridine for 30 min and isolated by BDC or poly(T)-cellulose chromatography decays with an approximately first-order kinetics, with a half-life of about 2 days (Fig. 6*A*). mRNA labeled with [^{3}H]uridine for 24 hr and isolated by BDC chromatography also appears to decay exponentially with a half-life that, by extrapolation of the curve, was estimated to be about 3 days (Fig. 6*B*).

As a control for any possible side effects of the "cold" chase that might lengthen the life-time of the mRNA, HeLa cells were labeled for 24 hr with [^{3}H]uridine and suspended in fresh medium without added uridine or cytidine. Fig. 6*B* shows that the metabolic stability of the mRNA chased in this manner, isolated by either BDC or poly(T)-cellulose chromatography, is close to that obtained after "cold" chase.

DISCUSSION

The main observation reported here is that in HeLa cells cytoplasmic mRNA is endowed with a much greater stability than has been assumed up to now. The average half-life of the bulk of free polysome mRNA in these cells is estimated to be about 3 days.

The similarity of half-life between mRNA labeled for 30 min and for 24 hr suggests that there is no large subpopulation of unstable mRNA in HeLa cells. We do not know, however, whether the measured decay results from a random degradation of the various molecular species of mRNA, or whether there are different classes of mRNA with different half-lives. The existence of mRNA species less stable than

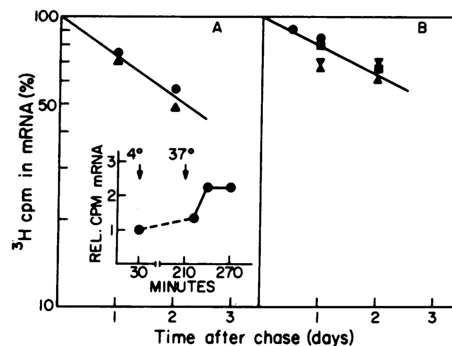

FIG. 6. Kinetics of decay of free polysome mRNA. (*A*) Equal samples of HeLa cells, labeled for 30 min with [^{3}H]uridine and subjected to the "cold" chase, were mixed, at different times, with the same amount of long-term [^{14}C]uridine-labeled cells; free polysome mRNA was extracted and purified by BDC (●) or poly(T)-cellulose(▲) chromatography. The amounts of [^{3}H]-uridine-labeled mRNA at different times have been normalized for recovery on the basis of the ^{14}C label. The zero-time value (determined after warming the culture to 37°) has been corrected: (*i*) for the continued arrival at the polysomes of mRNA labeled during the 30-min pulse, on the basis of the increment in labeled mRNA isolated on poly(T)-cellulose 1 hr after rewarming (*insert*), after subtraction of the small contribution of new synthesis from the labeled pools, estimated as described below [the increment measured relative to the amount at the end of the pulse would correspond to a mRNA pool equivalent to 12 min of synthesis, in good agreement with previous observations (21)]; (*ii*) for the continued synthesis from the labeled uridine and cytidine nucleotides; the fractional increase due to these nucleotides was estimated from the ratio of the integral of the UTP and CTP pool labeling in the 3-hr interval after warming (Fig. 1*B*) to that in the interval 0–30 min (estimated from Fig. 1, ref. 26). No account was taken of the 35% depression in rate of RNA synthesis; its effect would be small and in any case would increase the estimate of mRNA stability. (*B*) Equal samples of HeLa cells were labeled for 24 hr with [^{3}H]uridine and subjected either to the "cold" chase, with the mRNA being isolated by BDC (●,■), or to resuspension in fresh medium at 37°, with the mRNA being isolated by BDC(▼) or poly(T)-cellulose (▲) chromatography. The data have been normalized for recovery of the mRNA on the basis of long-term [^{14}C]labeled mRNA from cells added as internal control.

bulk RNA is hinted at by the somewhat shorter lifetime measured for the mRNA labeled for 30 min, as compared to that labeled for 24 hr. One component of this shorter-lived mRNA fraction is possibly the histone messenger (5, 27). Likewise, the analysis of the decay of mRNA has not been extended for a long enough time to exclude the existence of a fraction of the mRNA population with a half-life considerably longer than the average. Although there is a slight suggestion of a leveling off of the decay curve of mRNA labeled for 24 hr, further work is needed to verify this point.

The considerable stability shown here for the bulk of free polysome mRNA in a rapidly growing mammalian cell line provides a striking contrast to the situation in bacteria, where the majority of mRNA is short-lived (28). This difference presumably underlies a fundamental difference in regulation of genetic expression in the two types of organisms. While in bacteria this regulation operates mainly at the level of transcription (29), in animal cells translational mechanisms of control are likely to play a prominent role.

The average half-life of cytoplasmic mRNA previously estimated in HeLa and L cells with actinomycin D (4, 5) (3–4 hr) was much shorter than that measured here. This result implies that the assumption that the polysome breakdown caused by actinomycin D is the consequence of normal degradation of pre-existing mRNA was not correct. The actual mechanism of drug-induced polysome decay is not known. This polysome breakdown may represent only one aspect of the general cellular deterioration that actinomycin induces in HeLa cells (6–8). An alternative explanation is that the cells may possess a labile RNA or protein factor needed for polysome stability, whose continued synthesis or function is inhibited by the drug.

The new chase procedure described here has allowed the analysis of the stability of mRNA labeled during a short [^{3}H]uridine pulse. The mechanism by which the intracellular pools become depleted of radioactivity during the "cold" chase has not been studied in detail, although the experimental evidence indicates that expansion of the pools and release of the labeled precursors into the medium, presumably after dephosphorylation, play a role. With the exception of a transient depression by 35% of the rate of net RNA synthesis, no side effects of the "cold" chase were observed. A possible cause for this depression are the changes induced by the excess of uridine in the intracellular concentration of several nucleotides, which persist for at least 5 hr after rewarming the culture. The 3-hr recovery period may represent the time needed for the cells to adapt to these pool changes. The chase procedure described here provides a valuable alternative to the use of inhibitors of RNA synthesis for the study of the stability or processing of RNA species in animal cells under more physiological conditions.

In the present work, chromatography on BDC columns, first used for purification of bacterial mRNA (16), has been applied with success to isolation of mammalian mRNA. The observation that messenger of animal and bacterial cells behaves alike on this resin may point to a common property of mRNA of all organisms. The recovery of mRNA labeled with [^{3}H]uridine or [^{14}C]uridine for 30 min or 24 hr was slightly better (about 15%) after poly(T)–cellulose than after BDC chromatography. In the light of this observation, the finding that the BDC consistently gave a better yield of mRNA (6–9%) than the poly(T)–cellulose from the 1-day chased samples, and even better (16–18%) from the 2-day chased samples, may be significant as a possible indication of changes occurring in the mRNA with ageing, which affect its retention on poly(T)–cellulose columns.

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