*RNA STABILITY AND PROTEIN SYNTHESIS IN RELATION TO THE DIVISION OF MAMMALIAN CELLS**

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Experimental determinations of the stability of messenger RNA[1] (mRNA) provide a useful parameter to aid in understanding the mechanisms of transcription and translation of genetic information into functional protein. A common method for estimation of mRNA stability in mammalian cells is to inhibit RNA synthesis with a drug (e.g., actinomycin) and then to compare the resulting rate of protein synthesis or rate of polyribosomal breakdown with that of a drug-free control.[2, 3] A major limitation of such studies is that the mean lifetime thus estimated is averaged over a large number of different mRNA species of perhaps widely different lifetimes. One method of limiting attention to a specific mRNA/protein complex is to work with a highly specialized cell capable of synthesizing a limited number of proteins. Thus, the rabbit reticulocyte system has been employed to provide information on the stability of hemoglobin mRNA.[4, 5]

In this paper we report results obtained by a different method in which some specificity is obtained by observing a unique cell function (in this case, cell division) and in which highly precise timing is attained by use of the methods of life-cycle analysis.[6] A pattern of drug inhibition is chosen to produce a population of premitotic cells which have a full complement of mRNA's but not of the proteins necessary for division. The cells are held in this state with both protein and RNA synthesis inhibited for increasing periods of time to allow loss of messengers to occur. They are then released into a medium in which protein synthesis is permitted but RNA synthesis is still blocked. The fraction of cells able to complete mitosis under these conditions is taken to be a measure of survival of mRNA through the period of block. Employing this assumption to interpret the experimental data, we find that the mean lifetime of mRNA coding for protein essential for division of the Chinese hamster ovary cell is approximately 2.9 hr.

Materials and Methods.—Propagation of Chinese hamster ovary (CHO) cells in F-10 medium and partial synchronization of cells with thymidine have been described in detail elsewhere.[6] Cells were routinely examined for PPLO contamination with the agar of Chanock *et al.*[7] No PPLO were observed.

Cell concentrations were determined by counting aliquots of 2 ml of cell suspension diluted with 8 ml of isotonic saline in an electronic cell counter[8] using a 100×100-μ aperture. Since total counts ranged from 0.3 to 2.0×10^5, the counting statistical errors (1σ) were always less than 0.6 per cent. Reproducibility of results indicated that a precision of better than 1 per cent was usually obtained.

Synthesis of RNA and protein was determined by measuring incorporation of H^3-uridine and C^{14}-leucine into trichloroacetic acid-precipitable material.[9]

C^{14}-Leucine (201 mc/mM) and H^3-uridine (4.4 c/mM) were purchased from Schwarz BioResearch, cycloheximide (Acti-dione) was purchased from The Upjohn Company, and actinomycin D was a gift from Merck, Sharp and Dohme.

Results.—Conditions of block and release from cycloheximide: Cycloheximide (CHM) was selected to block protein synthesis because its effect is reversible,[10] and further, it reduces the rate of breakdown of polyribosomes which accompanies protein synthesis.[11, 12] In contrast, puromycin brings about an accelerated release of ribosomes from polyribosome aggregates.[5, 10, 12]

Since cell division was chosen as the fiducial mark, it was first necessary to establish the minimum concentration of CHM producing the maximum inhibition of division. CHM was added at concentrations ranging from 0.1 to 50 μg/ml to either random or partially synchronized cultures of CHO cells. The increase in cell number essentially ceased within 1 hr after addition of 2 μg/ml. Higher concentrations gave identical results, whereas lower concentrations produced an initial reduction in rate of division within 1 hr, followed by an almost complete cessation of division about 2.5 hr later.

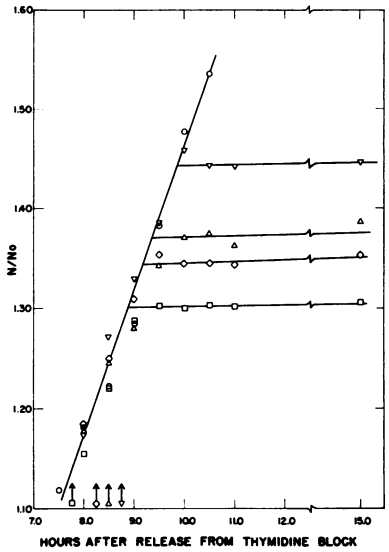

FIG. 1.—Determination of time interval between cycloheximide addition and cessation of cell division. Cycloheximide was added to cultures of partially synchronized CHO cells to a final concentration of 2 μg/ml at the times indicated. Cell concentrations were determined with an electronic particle counter.

The time delay between drug addition and cessation of division can be accurately measured by frequent counting of samples from random cultures. If partially synchronized cultures are employed,[6, 13] sharper delineation of the point of action of the drug is possible since the rate of change of cell concentration is 3–4 times as great. Partial synchrony was therefore induced by maintaining cells in 10 mM thymidine for 9 hr and resuspending in normal F-10 medium. CHM (2 μg/ml) was added to the cultures at varying times during the early stages of the parasynchronous wave. About 1 hr after CHM addition, cell division essentially ceased (Fig. 1), and over the ensuing 24 hr a further increase in cell number of only 2 per cent was observed. From the data in Figure 1, the mean lag time between CHM addition and subsequent inhibition of division was 59 $\pm$ 5 min. A value of 63 $\pm$ 5 min was obtained when randomly growing cells were employed.[14] These observations can be interpreted as identifying in the life cycle of the cell a point *C*, located about 60 min prior to division; in the presence of CHM, cells younger than *C* are prevented from dividing, while cells older than *C* are able to continue through the division process.

Optimal conditions for reversal of the CHM effect should produce a minimum time interval between removal of the drug and resumption of cell division, and a minimum perturbation in the pattern of cell growth upon resumption of division. These conditions were met by washing the cells twice in resuspension medium, resuspending the cells in "conditioned" medium (i.e., cell-free medium which had previously supported cell growth to 0.75–1.5 $\times$ 10^5 cells/ml; neither freshly prepared nor spent medium worked as well), and conducting all operations at 37°.

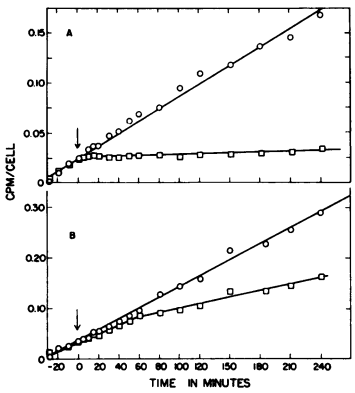

FIG. 2.—(*A*) Incorporation of C^{14}-leucine (0.2 μc/ml) into CHO cells growing in F-10 medium from which leucine had been omitted. (*B*) Incorporation of H^3-uridine (2 μc/ml) into CHO cells growing in leucine-free F-10 medium. Cycloheximide was added at $t = 0$ to a final concentration of 2 μg/ml. In both (*A*) and (*B*), control is represented by circles, and the cycloheximide-treated culture is plotted with squares.

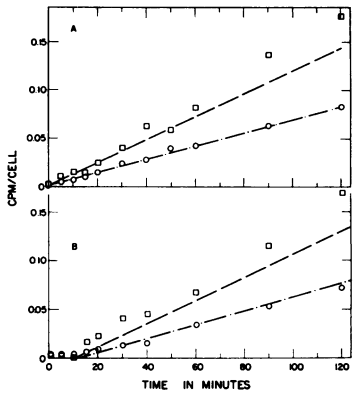

FIG. 3.—Incorporation of H^3-uridine and C^{14}-leucine into CHO cells following removal of cycloheximide after incubation periods in the drug of either 2 hr (*A*) or 5 hr (*B*). Conditions for incorporation are those described in Fig. 2. Squares represent uridine incorporation and circles represent leucine incorporation. The broken lines represent the rate of incorporation in drug-free controls and are not fit to the data points shown.

Effects of cycloheximide on RNA and protein synthesis: To obtain an estimate of the effect of CHM on protein synthesis, the incorporation of C^{14}-leucine was followed in suspension cultures of CHO cells in leucine-free F-10 medium, with and without the drug. From Figure 2*A*, it is apparent that leucine incorporation is grossly reduced but not shut off completely within the first few minutes after addition of CHM. (The very small amount of residual incorporation may explain the earlier observation that cells inhibited with CHM showed an increase in number of about 2% over a 24-hr period.)

Uridine incorporation was slightly reduced during the first 60 min of incubation in CHM (Fig. 2*B*); the effect became more pronounced thereafter, so that after 4 hr the rate of uridine incorporation had fallen to about 60 per cent of control value. These data for CHM effects on macromolecular synthesis in CHO cells are in good agreement with results obtained in L cells.[18]

Leucine and uridine incorporation was also followed in cultures in which CHM had been removed ($t = 0$) after periods of incubation in the drug of 2 hr (Fig. 3*A*) and 5 hr (Fig. 3*B*). For ease of comparison, the rates of incorporation of uridine and leucine in the noninhibited control cultures are presented as broken lines. Note that whereas RNA and protein synthesis began immediately after release in cultures inhibited for 2 hr, resumption of synthesis was delayed by 5–15 min in cells maintained in CHM for 5 hr.

Effects of variable incubation periods of random cultures in cycloheximide: In preliminary experiments it appeared that there was a difference between the time required for resumption of division in cells held for long periods of time in CHM and those maintained in the drug for only brief periods. CHM was therefore added to a large culture of randomly growing cells, and at varying times thereafter aliquots were removed and released from block (Fig. 4).

If the action of CHM were completely reversible, one should expect resumption of cell division 1 hr after drug removal. Cells closer in time to division have already divided in the presence of CHM, resulting in an emptying of that 1-hr portion of the life cycle preceding division. Cells maintained in CHM for periods up to 2.5 hr did indeed resume division about 70 min after drug removal, in agreement with our prediction. Growth curves following block removal were not significantly different from a smooth exponential function (i.e., there was no evidence of partial synchronization), implying that each cell resumed progress along the life cycle from a point at which it was stopped by the block. However, cells held in CHM for periods of 3 hr or longer were delayed about 130 min before resuming division. Since the delay did not increase with incubation periods in CHM in excess of 3 hr, it was not due to cumulative damage by the drug. Further evidence against cumulative damage was provided by the fact that once division resumed, even in the cultures delayed for 130 min, the resultant division rate was equivalent to that of the control. During prolonged CHM blockade, either all cells are delayed an additional 60 min before resuming division or, alternatively, a specific segment of the population (between 70 and 130 min prior to division) is incapacitated and fails to resume division.

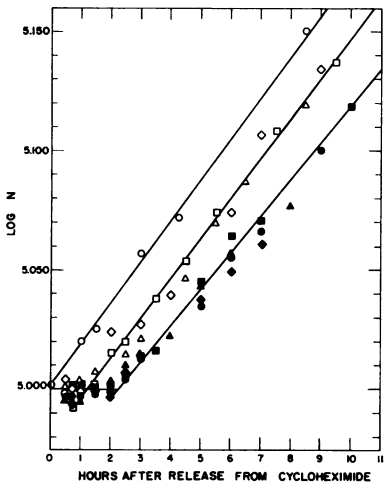

FIG. 4.—Resumption of division of CHO cells held in 2 μg/ml of cycloheximide for varying periods of time prior to removal of the drug at $t = 0$. The cultures were held in cycloheximide for the following periods: ○, drug-free control; □, 1.5 hr; ◇, 2.0 hr; △, 2.5 hr; ■, 3.0 hr; ●, 4.0 hr; ▲, 5.0 hr; and ◆, 8.0 hr.

Variable periods of incubation of synchronized cells in cycloheximide: The rate of loss of function can be studied in more detail by limiting the number of cells capable of division. We have previously reported that the actinomycin D (AMD) time marker (i.e., the end of RNA synthesis essential for division) is located 1.9 hr prior to division in the CHO cell.[6] If both CHM and AMD are added simultaneously, the population can be divided into three distinct categories: (1) those cells which have passed *C* and have a full complement of all proteins essential for division; (2) those cells which have passed *A* (the AMD block point) but not *C* and have a full complement of RNA but have not yet completed synthesis of all proteins necessary for division; and (3) those cells which have not reached *A* and are lacking both RNA and protein essential for division.

Cells of class (1) will proceed through division even in the presence of both drugs and will leave a 1-hr "gap" in the population distribution. The other two classes will be stopped so long as the effects of drugs continue. If only the CHM block is released, the AMD will continue to prevent cells of class (3) from resuming synthetic activities and only those cells of class (2) which retain the ability to synthesize protein will be able to continue through division. Furthermore, cells of class (2) can only resume translation to the extent that they retain functional messenger, since AMD continues to inhibit renewed RNA synthesis. The number of

cells in this class can be calculated from the distribution of cell ages at time of block as deduced from the growth curve of the control culture. For short block times, this is the maximum number of cells which should divide. As the block time is increased, the dividing fraction declines in proportion to loss of function.

Random cultures could be used for such an experiment, but the number of trapped cells can be appreciably increased with partially synchronized cultures. We have previously shown that the thymidine synchronization procedure does not grossly distort the timing of these parts of the life cycle of interest here.[6] Accordingly, cells were partially synchronized with the aid of 10 mM thymidine,[13] and CHM and AMD blocks were applied simultaneously at the beginning of the division wave. After varying times in block, the cells were resuspended in normal medium, thus releasing the CHM block. Actinomycin in the concentrations used here (2 μg/ml) irreversibly inhibited RNA synthesis[19] and continued to be effective even after removal from the medium. In agreement with expectation, the maximum number of cells dividing under these conditions approached but never exceeded the number of cells calculated to be trapped between markers *A* and *C*. In Table 1 (expt. A) and in Figure 5 (*solid points*), the fraction of trapped cells that divided is presented as a function of time in both CHM and AMD. This fraction dropped to 50 per cent in 2.9 hr.

To investigate the possibility of synergistic side effects during the double block, the following variation of the above experiment was performed. Partially synchronized cells were blocked with only CHM for varying periods of time and then released into medium containing AMD. The number of cells that divided was again expressed as the fraction of those trapped between *A* and *C*, and the results are presented in Table 1 (expts. B, C, and D) and in Figure 5 (*open points*). The effect is indistinguishable from that observed in the preceding experiment in which AMD was present during the preliminary block. This result appears to eliminate the possibility of undesirable side effects from AMD, and more interestingly, it demonstrates that even though RNA synthesis continues (Fig. 2*B*), no mRNA functional for division has been synthesized during the CHM block. Such synthesis would have permitted cells of age less than *A* to cross that marker and would result in an increase in number of cells trapped between *A* and *C*. The fraction dividing (Fig. 5) would then have exceeded 100 per cent.

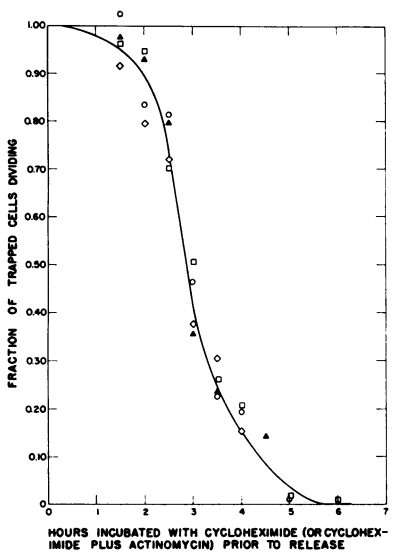

FIG. 5.—Fraction of expected number of cells dividing as a function of time in cycloheximide blockade. Solid points represent cells incubated in cycloheximide and actinomycin simultaneously (final concentration of both drugs, 2 μg/ml) and then released into medium without either drug. Open points represent cultures incubated in cycloheximide only prior to release into medium containing actinomycin.

Discussion.—In the present report synthesis of protein has been reversibly inhibited by CHM, allowing examination of cessation and resumption of division upon addition and removal of the drug. The CHM block was found

TABLE 1

FRACTION OF CELLS RETAINING THE CAPACITY TO DIVIDE AFTER VARYING INTERVALS OF INHIBITION BY CYCLOHEXIMIDE

Incubation period (hr) in cycloheximide plus actinomycin	Incubation period (hr) in cyclo-heximide	Cell increase (%) following release into drug-free medium	Cell increase (%) following release into actinomycin	Cell increase (%) expected from control	Cell increase observed to cell increase expected
Expt. A					
1.5	...	10.9	...	11.2	0.97
2.0	...	10.4	...	11.2	0.93
2.5	...	8.9	...	11.2	0.80
3.0	...	4.0	...	11.2	0.36
3.5	...	2.6	...	11.2	0.23
4.0	...	2.1	...	11.2	0.19
4.5	...	1.6	...	11.2	0.14
Expt. B					
...	1.5	...	11.2	10.7	1.04
...	2.0	...	8.9	10.7	0.83
...	2.5	...	8.7	10.7	0.81
...	3.0	...	5.0	10.7	0.47
...	3.5	...	2.4	10.7	0.22
...	4.0	...	2.1	10.7	0.20
...	5.0	...	0.5	10.7	0.03
Expt. C					
...	1.5	...	12.2	12.7	0.96
...	2.0	...	12.0	12.7	0.95
...	2.5	...	8.9	12.7	0.70
...	3.0	...	6.4	12.7	0.50
...	3.5	...	3.3	12.7	0.26
...	4.0	...	2.6	12.7	0.21
...	5.0	...	0.5	12.7	0.03
...	6.0	...	0.5	12.7	0.03
Expt. D					
...	1.5	...	11.4	12.5	0.91
...	2.0	...	9.9	12.5	0.79
...	2.5	...	8.9	12.5	0.72
...	3.0	...	4.7	12.5	0.38
...	3.5	...	3.8	12.5	0.30
...	4.0	...	1.9	12.5	0.15

to be fully reversible if released within 2.5 hr, and in this case division resumed after the expected 60-min delay. Longer blocks resulted in a nearly constant additional delay period of about 1 hr before normal progression in the life cycle was resumed, suggesting that the "survival" point in the life cycle had shifted back from *C* (completion of protein synthesis) to *A* (completion of RNA synthesis). The rate of loss of function by those cells between *A* and *C* was measured by using AMD to prevent additional cells from crossing *A*. It was found that the fraction of *A*–*C* cells that were able to continue through division in the presence of AMD was 100 per cent for short-duration CHM blocks, but that after 2.9 hr it had fallen to 50 per cent and was essentially zero after 5 hr in CHM.

There is excellent agreement on estimated lifetime of the unstable species of about 3 hr, whether obtained with random cells plus CHM (Fig. 4), or with synchronized cells plus both CHM and AMD (Fig. 5). This is a unique time-dependent loss of function by a small group of late G_2 cells. For the rest of the population, CHM is apparently completely reversible, as judged by resumption of cell division at rates equivalent to control values (Fig. 4) and by return to normal rates of protein and RNA synthesis shortly after reversal of prolonged CHM blocks (Fig. 3*B*). Thus, generalized losses (such as breakdown of the enzymatic and ribosomal machinery)

cannot account for the loss of function of A–C cells. It seems clear, therefore, that the effect is peculiar to the A–C portion of the life cycle. The feature that distinguishes A–C cells from the rest of the population is that they are the *only* cells that have completed synthesis of all RNA species essential to division. In view of the known instability of mRNA, it is likely that the material undergoing a loss of function with increasing time in CHM is one or more species of mRNA containing information for the essential division proteins.

Since similar results were obtained whether AMD was added only at the time of release from CHM or was added simultaneously with CHM (preventing synthesis of RNA during CHM block), it follows that no functional mRNA accumulated in the presence of CHM alone. That some RNA synthesis continues in the presence of inhibitors of protein synthesis is clear from the work of Latham and Darnell[21] on puromycin and from the isotope incorporation data of Figure 2*B* for CHM. However, this continuing gross synthesis does not lead to accumulation of specific mRNA's required by the premitotic cells studied here.

If the rate of mRNA destruction *in vivo* is related to rate of translation in noninhibited cultures, the lifetime for division-associated mRNA species may be less than the 2.9-hr value reported here. In any event, it is evident that the messenger(s) coding for final protein(s) essential to division is functionally stable only during the portion of the life cycle during which it is required. The value presented here for stability of mRNA pertaining to division need not be representative of other species of mRNA in the CHO cell. In fact, when leucine incorporation was followed in randomly growing cultures of CHO cells with and without actinomycin, the calculated time at which protein synthesis in actinomycin-treated cultures had dropped to 50 per cent of the control was about 4.5 hr.[9] The 4.5-hr value provides an estimate of mean lifetime of a large number of different mRNA species operative at all stages of the life cycle of the CHO cell.

Our data do not indicate conclusively the fate of those cells trapped between the A and C markers that lose mRNA during prolonged CHM block. They might be "set back" in development, necessitating resynthesis of the decayed mRNA species before resumption of division. In this case, unless cells at all stages of the life cycle are set back to the same extent, one should expect to see in Figure 4 an increased division rate at the time these delayed cells reach the end of mitosis, since they would then be accompanied by cells which were stopped before the A marker. This effect is not observed, but it is of small magnitude and might not be detectable. An alternative explanation seems more attractive: after completion of synthesis of RNA required for division, cistron(s) containing information for essential division protein(s) may become inaccessible for further transcription. Upon reversal of prolonged CHM blocks, the cells trapped between the A and C markers would be unable to synthesize the proteins required for division due to loss of mRNA and would further be unable to retranscribe the last species of mRNA owing to inaccessibility of a specifically related segment of the genome. Thus, these cells would not divide upon removal of CHM.

Our observation that inhibition of synthesis of essential division proteins by cycloheximide may be accompanied by an inhibition of synthesis of a closely associated RNA species (presumably messenger) is in agreement with the model of control of genetic transcription at the translational level recently proposed by Cline and

Bock.[22] Our results contain no direct evidence of mechanisms, but repression of synthesis of a specific fraction of RNA in the presence of a nearly normal rate of total RNA synthesis (Fig. 2*B*) may suggest a specialized control mechanism.

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[1] The following abbreviations are used: mRNA = messenger ribonucleic acid; CHM = cycloheximide; AMD = actinomycin D; *A* time marker = time of synthesis of the last RNA species essential to division, located at 1.94 hr before division; *C* time marker = time of synthesis of the last essential protein species, located at 1 hr before division; and CHO cell = Chinese hamster ovary cell.

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[14] In similar experiments involving puromycin, we obtained a value of 36 ± 5 min for the lag time between puromycin addition and cessation of division.[6] While more recent experiments tend to minimize the difference, the discrepancy between these two values still appears to be real and may possibly represent a more rapid inhibition of functional protein synthesis in puromycin-treated cells. It is known that puromycin produces an immediate breakdown of polyribosomes,[5, 10, 12] which cycloheximide does not.[11, 12] Furthermore, puromycin prevents completion of developing polypeptides.[15, 16] In this regard the data are in agreement with the interpretation recently proposed by Taylor,[17] which suggests that cessation of biosynthesis of *functional* protein in the presence of puromycin is almost immediate despite the fact that measurable isotope incorporation persists for some time. The mechanism of action of cycloheximide is not known. We are presently investigating this highly reproducible discrepancy in time of action of the two drugs.

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