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*TRANSLATIONAL INHIBITION IN MITOTIC HELA CELLS**

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Communicated by Alfred Gilman, September 23, 1965

Macromolecular synthesis during the life cycle of the mammalian cell undergoes periodic fluctuations, among the most striking of which is the cessation of RNA synthesis and the marked decrease in rate of protein synthesis as the cell enters mitosis.[1-4] During the brief period of mitosis—which may be extended temporally by arresting cells in metaphase—suppression of macromolecular synthesis persists and even extends to control over synthesis by an invading viral genome.[5] The rapid decrease in amino acid incorporation in mitotic cells and the marked reduction in numbers of polyribosomes observed in cells arrested in metaphase[6] cannot be accounted for by the cessation of RNA synthesis, since pre-existing messenger RNA has been shown to have a half life significantly in excess of the duration of mitosis.[7] Thus, there is obviously a direct inhibition of protein synthesis. This communication describes studies on metaphase cells, utilizing an amino acid incorporation system similar to that of Nirenberg and Matthaei,[8] which suggest the mechanism of inhibition of protein synthesis in cells entering mitosis, and provide an explanation for the inhibition of viral synthesis in the metaphase-arrest cell.[5]

Materials and Methods.—Metaphase cells: HeLa S3 cells were grown as monolayers in attachment solution[9] minus Ca^{++}, supplemented with 3% fetal bovine serum. To obtain large populations of cells in metaphase, a combination of the preferential harvesting technique, using low Ca^{++} medium,[3, 10] and the thymidine synchronization technique[11, 12] was used. Attachment solution containing 2 mM thymidine was added to nearly confluent monolayers and the cells were incubated 24 hr. At the end of this period, the medium was replaced with thymidine-free medium After 8 hr, thymidine (2 mM) was again added and the cells were incubated 14 hr. The medium

was then replaced with thymidine-free medium. After an additional 10–11-hr incubation, cells in metaphase, which are tenuously attached to the glass in low Ca^{++} medium,[10] were collected by gently shaking the bottles. Harvested metaphase populations, containing some cells in anaphase, were immediately chilled in an ice-salt bath to prevent escape from mitosis. Over 95% of those cells detached by shaking (about 50–60% of the total cell population) were in metaphase or anaphase, as judged by phase-contrast microscopic examination. Those cells not detached by the above procedure were scraped from the glass and used as control interphase cells. Where indicated, some experiments used HeLa cell cultures treated with vinblastine sulfate to provide large numbers of metaphase-arrest cells as reported.[5] Vinblastine-exposed cells not recruited into metaphase-arrest served as control-interphase cells.

Cell fractionation: Metaphase and control interphase cells were washed in phosphate-buffered saline and resuspended to a density of 4×10^7/ml in EtSH buffer (tris-HCl, pH 7.4, 10 mM; KCl, 10 mM; Mg $(CH_3COO)_2$, 1.5 mM; 2-mercaptoethanol, 6 mM). The cells were swollen in this buffer for 10 min and then broken with five strokes of a tight-fitting Dounce homogenizer. Both interphase and metaphase cells are completely disrupted, and less than 2% of total nuclei are broken using this procedure. Nuclei and large debris were removed by centrifugation at 800 *g* for 10 min. The resulting supernate, referred to as crude cytoplasmic extract, was either immediately used or separated into ribosomal and supernatant (*S*100) fractions by centrifugation at 105,000 *g* for 2 hr at 2°C. The *S*100 fraction was stored at −70°C until use. Ribosomes were purified by treatment with 0.5% sodium desoxycholate and precipitation with 0.05 *M* $MgCl_2$.[13] This procedure retains all polyribosomes intact. Approximately 12 OD_{260} units of ribosomes, or about 1 mg per 4×10^8 cells, was isolated from both interphase and metaphase cell populations. Equal amounts of ribosomes were obtained from each population as judged by optical density measurement of the extracted 28*S* RNA.

In vitro incorporation: The reaction mixtures contained, in a final volume of 1.0 ml, the following (in μmoles): tris-HCl, pH 7.4, 100; KCl, 50; Mg $(CH_3COO)_2$, 14; 2-mercaptoethanol, 6; adenosine-5′-triphosphate, 1.0; guanosine-5′-triphosphate, 0.03; sodium phosphoenolpyruvate, 7.5; pyruvate kinase, 10 μg; interphase or metaphase cell *S*100 fraction, 0.30 ml; interphase or metaphase cell ribosomes, 0.30 mg; L-phenylalanine-C^{14} (320 μc/μM), 1.0 μc. When specified, polyuridylic acid, 50 μg, was added. The mixtures were incubated for various periods of time at 37°C and the reactions terminated by the addition of 4 ml cold 10% trichloroacetic acid containing 20 mg/ml casamino acids and 0.5 mg bovine serum albumin as carrier The precipitates were centrifuged, solubilized in 1 *N* NaOH, reprecipitated, plated onto Millipore filters, and washed extensively with 5% TCA. Radioactivity was measured to an accuracy of ±3% on a low-background Nuclear-Chicago gas-flow counter operating at 30% counting efficiency.

Trypsin treatment: The ribosomes to be treated were suspended in 1.0 ml EtSH buffer containing the appropriate amount of 2 × crystallized trypsin, and incubated for 30 min at 33°C. The suspensions were chilled, centrifuged 2 hr at 105,000 *g*, and the ribosomal pellet resuspended in EtSH buffer. The ribosomes were precipitated with 0.05 *M* $MgCl_2$, and again suspended in EtSH buffer.

Reagents: Tris-HCl (pH 7.4), adenosine-5′-triphosphate, disodium; guanosine-5′-triphosphate, disodium; sodium phosphoenolpyruvate, pyruvate kinase, and thymidine were obtained from Sigma Chemical Co. Polyuridylic acid, ammonium salt, was obtained from Miles Laboratories, and 2 × recrystallized trypsin was from Worthington Biochemical Corp. Distillation Products Industries supplied the 2-mercaptoethanol. Sodium desoxycholate was obtained from Difco Laboratories. L-arginine-C^{14} and L-phenylalanine-C^{14} (uniformly labeled) were from New England Nuclear Corp.

Results.—Amino acid incorporation by crude cytoplasmic extracts of interphase and metaphase cells: Figure 1 shows the results of a representative experiment in which 2μc of L-arginine-C^{14} (240 μc/μM) was added to crude cytoplasmic extracts of vinblastine-interphase and metaphase-arrest cells, and the radioactivity of acid-insoluble material determined as a function of time at 37°C. The rate of amino acid incorporation into acid-insoluble form observed in interphase cell homogenates is typical for this type of preparation,[14] and is seen to be about three times more rapid than that achieved by extracts of metaphase cells. Both rates of incorporation

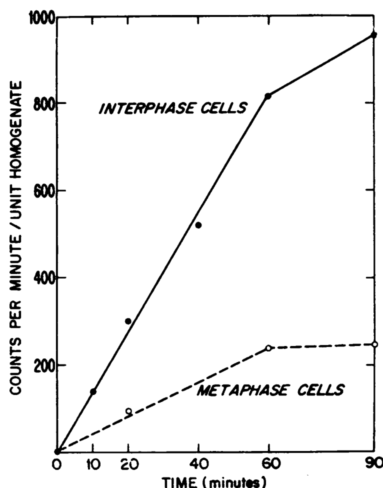

FIG. 1.—Incorporation of L-arginine-C^{14} by cytoplasmic extracts of vinblastine-interphase and metaphase-arrest HeLa cells. Populations of interphase and metaphase HeLa cells were suspended to densities of 4×10^7 cells/ml and crude cytoplasmic extracts prepared as described. L-arginine-C^{14} at 1 μc/ml (240 μc/μM) was added and the mixtures were incubated at 37°C. At various times, 0.2-ml aliquots were removed and acid-precipitable radioactivity was determined. A unit of homogenate consisted of the crude extract from 8×10^6 cells and contained about 2.7 mg total protein.

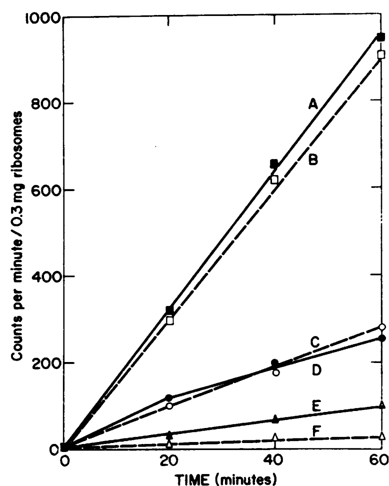

FIG. 2.—Polyuridylic acid-directed L-phenylalanine-C^{14} incorporation by combinations of interphase and metaphase ribosomal and *S*100 fractions. Each reaction mixture, as described in *Materials and Methods*, contained 0.90 mg preincubated ribosomes, 1 μc L-phenylalanine-C^{14} (320 μc/μM), and, when specified, 50 μg polyuridylic acid. Incubation was at 37°C. (*A*) Interphase ribosomes + interphase *S*100 + poly U, (*B*) interphase ribosomes + metaphase *S*100 + poly U, (*C*) metaphase ribosomes + metaphase *S*100 + poly U, (*D*) metaphase ribosomes + interphase *S*100 + poly U, (*E*) interphase ribosomes + interphase *S*100 − poly U, (*F*) metaphase ribosomes + metaphase *S*100 − poly U.

remain linear for about 60 min, and then decrease. A similar experiment with extracts from normal interphase and mitotic cells incubated for 60 min at 37°C with L-arginine-C^{14} revealed acid-insoluble material amounting to 653 cpm and 205 cpm, respectively.

These *in vitro* results parallel those obtained by Prescott and Bender in autoradiographic studies[1] and by Robbins and Scharff in whole cells.[6] The results show that the low rate of protein synthesis observed in intact metaphase cells is reflected precisely in crude homogenates prepared from them. Consequently, experiments were designed to determine which of the components involved in protein synthesis is rate-limiting in the mitotic cell.

Ribosomes—The rate-limiting component in mitotic cell protein synthesis: Ribosomes from interphase and metaphase cells were preincubated in an energy-generating system with *S*100 fraction for 30 min to break down polyribosomes and decrease background incorporation.[15] Synthetic messenger, in the form of polyuridylic acid ($S_{20,w}$ = 6.42) was then added, and L-phenylalanine-C^{14} incorporation into poly-L-phenylalanine-C^{14} measured as a function of time. The results presented in Figure 2 demonstrate that the *S*100 fraction from metaphase cells (curve *B*) is, on the

average, equally as active as the interphase cell *S*100 fraction (curve *A*) in stimulating protein synthesis with interphase ribosomes. However, metaphase cell ribosomes, whether in combination with metaphase (curve *C*) or interphase (curve *D*) *S*100 fraction, are only about one third as active in protein synthesis as interphase cell ribosomes. Curves *E* and *F* represent endogenous incorporation of L-phenylalanine-C^{14} by interphase and metaphase ribosomal fractions, respectively, that have not been primed with polyuridylic acid. These results demonstrate that the primary rate-limiting component in *in vitro* protein synthesis in the metaphase cell system involves elements of the ribosomal population and not enzymic or ionic deficiencies in the *S*100 fraction.

Trypsin reactivation of metaphase cell ribosomes: The most profound intracellular reaction coincident with the decrease in rate of protein synthesis at the start of mitosis is disruption of the nuclear membrane. This singular event suggests a hypothesis to account for the decreased rate of protein synthesis during mitosis, namely, that breakdown of the nuclear membrane may be accompanied by release into the cytoplasm of nuclear material that binds to ribosomes and prevents their functioning in protein synthesis. To test this possibility, preincubated ribosomes from interphase and metaphase cells were incubated in the presence of varying amounts of twice-crystallized trypsin. The ribosomes were then washed free of trypsin, and amino acid-incorporating activity was measured by the standard assay. Figure 3 presents the results of one such experiment. Interphase ribosomes are

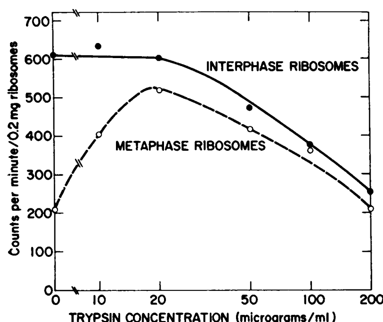

FIG. 3.—Trypsin reactivation of metaphase ribosomes. Aliquots containing 0.2 mg interphase or metaphase ribosomes were incubated with the appropriate amount of twice-crystallized trypsin as described. These ribosomes were then tested in the standard *in vitro* protein-synthesizing system, to which was added 50 μg polyuridylic acid and 1 μc L-phenylalanine-C^{14}. Each point represents the acid-precipitable counts obtained after an incubation period of 60 min at 37°C.

observed to retain maximal amino acid-incorporating capacity when treated with up to 20 μg trypsin/ml reaction mixture. In contrast, metaphase ribosomes, starting at a low rate of amino acid incorporation, regain most of their capacity following treatment with an optimal concentration of trypsin (20 μg/ml), and thereafter are about 85 per cent as active as interphase ribosomes. Above 20 μg trypsin/ml, ribosomes from metaphase and interphase cells behave similarly, losing protein-synthesizing capacity at approximately equal rates.

Discussion.—The experiments presented here demonstrate that the marked suppression of protein synthesis observed as cells enter mitosis is duplicated precisely in an *in vitro* amino acid-incorporating system when ribosomes from cells harvested in mitosis are substituted for ribosomes of interphase cells. Up to 85 per cent of the amino acid-incorporating activity of interphase cell ribosomes can be regained by ribosomes from mitotic cells if the latter are exposed to low concentrations of the proteolytic enzyme, trypsin. Thus, ribosomes of mitotic cells are inherently capa-

ble of supporting protein synthesis, but, as isolated from cells in metaphase, are prevented from doing so at the normal rate by the presence of a readily accessible trypsin-sensitive substance, possibly on their surface. The nature of this material, presumably bound protein, is presently unknown, although speculation that nuclear histones may be involved seems appropriate since histones (*a*) have been shown to be released into the cytoplasm when the nuclear membrane breaks down,[16] (*b*) inhibit protein synthesis by isolated ribosomes,[17] and (*c*) are among the most trypsin-sensitive proteins known, due to the specific cleavage of arginine and lysine bonds by this enzyme. These facts, while not conclusive, implicate histones, or some nuclear protein complex, as a specific and reversible translational inhibitor in mitotic cells. However, the presence of a minor contaminant of trypsin possessing other than proteolytic activity deserves consideration.

The intracellular events thought to initiate suppression of protein synthesis during mitosis may be logically attributed to the release of nuclear material into the cytoplasm following breakdown of the nuclear membrane at late prophase. This mitotic event is followed by a loss of most polyribosomes as visualized by electron microscopy and in sucrose gradient profiles by Robbins and Scharff.[6] The polyribosomes which remain in mitotic cells (approximately 25% the amount of interphase cell polyribosomes[6]) are presumably responsible for the incorporation observed in crude extracts from metaphase cells. The binding of trypsin-sensitive material to existing polyribosomes might adversely affect their integrity, reducing them to nonfunctional monomeric units; however, the dispersal of polyribosomes by another mechanism initiated upon breakdown of the nuclear membrane should be considered. Whether the cell activates a proteolytic system to recover the masked ribosomes at the end of mitosis remains unanswered. A simple model might relegate this responsibility to a small number of functional polyribosomes.

The trypsin-mediated reactivation of ribosomes from unfertilized sea urchin eggs reported by Monroy, Maggio, and Rinaldi[18] is similar to the reactivation of mitotic ribosomes described here. It is possible that protein coating of messenger RNA-ribosome complexes,[18] or the concomitant breakdown of polyribosomes,[6] or coating of monomers from mitotic cells may be mechanisms controlling translational expression in cells at specific critical periods in their life cycle. Although we have no evidence that potentially functional messenger RNA is in association with the protein-coated ribosomes, this possibility is considered unlikely because of the predominantly monomeric state of ribosomes in the mitotic cell.[6] Elucidation of the role of proteases as derepressors of translation would serve to extend our knowledge of control mechanisms in the cell. In this connection, the possible relationship of the trypsin-sensitive inhibitor associated with metaphase ribosomes to that reported by Hoagland *et al.*[19] as being active in *microsomal* preparations of normal liver is under investigation.

These data also provide insight into the mechanism of viral inhibition in the metaphase-arrest cell.[5] A previous study showed that virus attachment, entry into cells, and eclipse were normal in HeLa cells arrested in metaphase, but no viral RNA synthesis was initiated as long as the host cell remained in metaphase. However, upon removal of the spindle inhibitor, a significant number of cells escaped the metaphase block, and in all of these, viral RNA synthesis was self-initiating. In light of our present results, the failure to initiate viral RNA synthesis in metaphase-

arrest cells is interpreted as reflecting the low probability of viral messenger RNA's encountering functional ribosomes and forming the primary messenger RNA–ribosome complex—most ribosomes being blocked with protein. Upon infection of metaphase-arrest cells with Newcastle disease virus, even at high multiplicities, the nonfunctioning ribosomes apparently are not reaggregated into polyribosomes by viral messenger RNA since viral synthesis is held in abeyance until the cells escape the arrested state and uncoating of ribosomes is presumably initiated. In contrast, high multiplicities of the more cytopathic poliovirus can overcome the block to viral RNA synthesis in a significant fraction of the arrested cells. Whether poliovirus RNA, as messenger,[20] may preferentially pre-empt the trypsin-sensitive coat remains to be determined.

Summary.—Studies of mitotic HeLa cells in an *in vitro* system reveal that (*a*) ribosomes from metaphase or metaphase-arrest cells constitute the rate-limiting component in amino acid incorporation, (*b*) the relative rates of protein synthesis stimulated by ribosomes from interphase and mitotic cells, respectively, reflects precisely the rates observed *in vivo*, (*c*) the coating of ribosomes with a trypsin-sensitive material, presumably derived from the nucleus upon dissolution of the nuclear membrane at mitosis, is responsible for the observed translational inhibition, and (*d*) almost full activity can be restored to these coated ribosomes with gentle trypsin treatment which is thought to remove an inhibitor bound to the ribosomal surface. The possible role of nuclear histones as a translational inhibitor (repressor?) is considered, and an explanation offered for the previously observed inhibition of viral synthesis in the metaphase-arrest cell.

The authors would like to express their sincere gratitude to S. Penman, D. Summers, J. Warner, and J. Darnell for many helpful discussions.

* This investigation was supported by grant AI-03619-05 VR from the National Institutes of Health. Dr. Marcus is a Research Career Development Awardee of the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service (2-K3-GM-15, 461-05).

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