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Increased phosphorylation of elongation factor 2 during mitosis in transformed human amnion cells correlates with a decreased rate of protein synthesis

(cell cycle/control of protein synthesis/human protein data bases)

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ABSTRACT **Elongation factor 2 was identified in the two-dimensional gel patterns of asynchronous human amnion cells (AMA) by comigration with purified rabbit reticulocyte elongation factor 2 and by two-dimensional gel immunoblot analysis using a specific rabbit polyclonal antibody. In all, four polypeptides were identified corresponding to isoelectric focusing polypeptides 2713 (95.0 kDa), 2714 (94.8 kDa), 3727 (94.8 kDa), and 3702 (93.6 kDa) (listed in order of decreasing pI values) in the computerized comprehensive two-dimensional gel data base of human AMA proteins. The relative proportion of two of these variants (isoelectric focusing polypeptides 3727 and 3702), which are phosphorylated, increased dramatically during mitosis. Since phosphorylation is known to render elongation factor 2 inactive in translation, this observation may partly explain the decline in the rate of protein synthesis observed during cell division.**

When the eukaryotic cell undergoes mitosis the rate of protein synthesis declines to 20–30% of the interphase level (1–5). The molecular mechanisms underlying such a decline are at present unknown. It was found that protein synthesis elongation factor 2 (EF-2) can be phosphorylated *in vitro* and *in vivo* by the special Ca^{2+}/calmodulin-dependent EF-2 kinase (6–11). It was shown that phosphorylation of EF-2 by this kinase resulted in a complete inactivation of EF-2 in translation (7, 8, 10, 11). Furthermore, Redpath and Proud (12) have shown that phosphorylation of EF-2 inhibits translation of a natural mRNA in a cell-free system where initiation, elongation, and termination take place, suggesting that EF-2 phosphorylation may play a role in the control of translation (see also refs. 8 and 10). Here we have used high-resolution two-dimensional (2D) gel electrophoresis to investigate changes in the levels of synthesis of EF-2 variants throughout the cell cycle of transformed human amnion cells (AMA). Our results show that there is a dramatic and preferential increase in the relative proportion of two phosphorylated variants of EF-2 during mitosis, a fact that may partly explain the decline in the translation rate observed during cell division.

MATERIALS AND METHODS

Cells and Cell Synchrony. AMA cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal calf serum and antibiotics (penicillin at 100 units/ml and streptomycin at 50 μg/ml). Mitotic cells were obtained by mechanical detachment essentially as described by Terasima and Tolmach (13). Two 250-ml flasks containing 1–2 × 10^6 cells per flask were used. About 60% of the mitotic cells corresponded to metaphases, as determined by phase-contrast microscopy.

Labeling of Cells with [^{32}P]Orthophosphate and [^{35}S]Methionine. Mitotic and interphase AMA cells were labeled at 37°C for 25 min in 1 ml of phosphate-free medium (DMEM) containing 1 mCi of [^{32}P]orthophosphate (RISØ, Roskilde, Denmark; 1 Ci = 37 GBq) per ml. After labeling, the cells were washed in 0.9% NaCl and resuspended in lysis buffer (14). Cells were labeled with [^{35}S]methionine in 0.1 ml of minimal essential medium lacking unlabeled methionine (for short labeling periods only) and containing 10% (vol/vol) dialyzed fetal calf serum and 100 μCi of [^{35}S]methionine (Amersham, SJ204) (15).

2D Gel Electrophoresis. SDS/PAGE (15% running gel; 5% stacking gel) was carried out essentially as described by Laemmli (16). 2D gel electrophoresis [isoelectric focusing (IEF)] was carried out as described by Bravo (17). In short, the first dimension was performed in 130 mm × 1.2 mm 4% (wt/vol) polyacrylamide gels containing 2% (wt/vol) carrier ampholytes (1.6% pH 5–7, Serva; 0.4% pH 3.5–10, LKB) for 18 hr at 400 V. First-dimensional gels were equilibrated for 3 min at room temperature in 3 ml of equilibration solution [0.06 M Tris·HCl, pH 6.8/2% (wt/vol) SDS/100 mM dithiothreitol/10% (vol/vol) glycerol] (14). Gels were stored at 20°C until use. First-dimensional gels were applied to the second dimension with the aid of agarose solution (0.06 M Tris·HCl, pH 6.8/2% SDS/100 mM dithiothreitol/10% glycerol/1% agarose/0.002% bromophenol blue) (14). EF-2 was purified from rabbit reticulocytes as described (7).

The procedure for 2D gel immunoblotting has been described in detail (15).

RESULTS

EF-2 was identified in the 2D gel patterns of cellular proteins of asynchronous AMA cells (Fig. 1*A*) by comigration with purified rabbit reticulocyte EF-2 and by 2D gel immunoblot analysis using specific rabbit polyclonal antibodies kindly provided by A. Minin (Institute of Protein Research, Academy of Sciences of the U.S.S.R.). Four Coomassie brilliant blue-stained spots were detected in the purified EF-2 preparation (Fig. 1*B*) and these comigrated with [^{35}S]methionine-labeled AMA proteins termed IEF polypeptides 2713 (95.0 kDa), 2714 (94.8 kDa), 3727 (94.8 kDa), and 3702 (93.6 kDa) [Fig. 1*C*, AMA protein data base numbering system (18)]. All four proteins were recognized by the antibody in 2D immunoblots (Fig. 1*D*). It should be stressed that the first-dimension gel used to prepare the 2D blot shown in Fig. 1*D* was heavily overloaded to visualize the less-abundant variants IEF polypeptides 3727 and 3702. Under these conditions the more basic variants do not focus well (streak) and the ratio between the

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Abbreviations: IEF, isoelectric focusing; EF-2, elongation factor 2; 2D, two-dimensional.

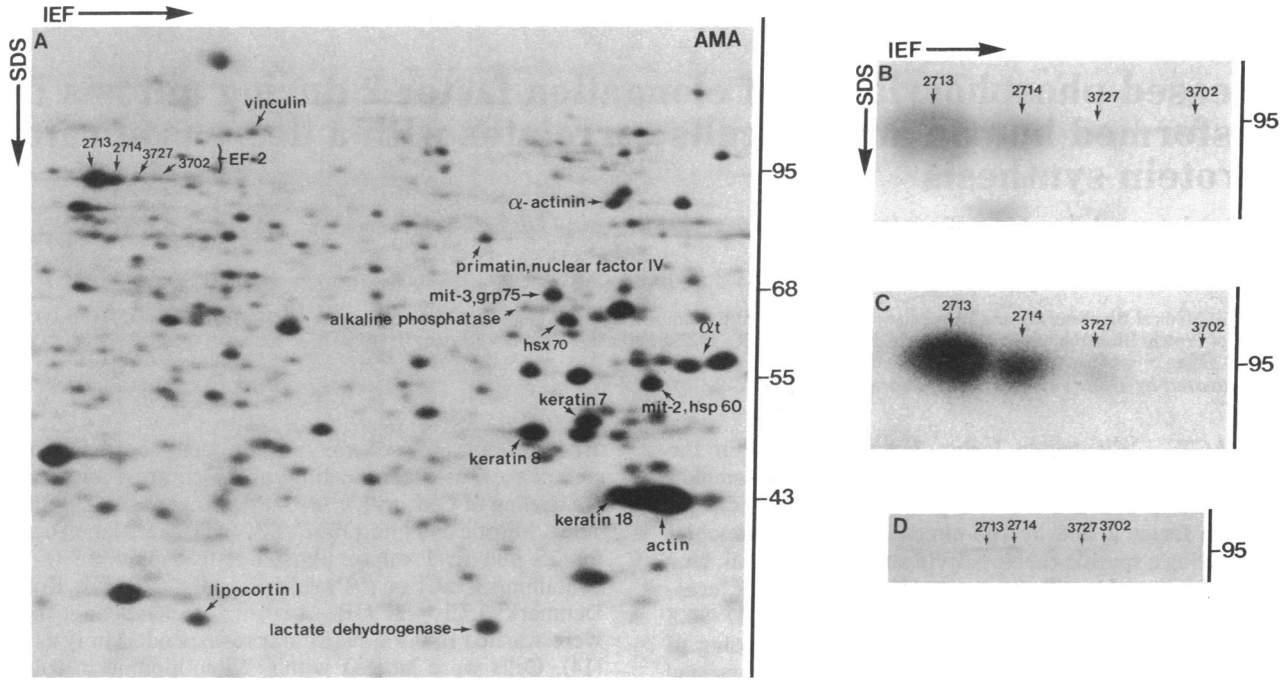

FIG. 1. Identification of EF-2 variants in 2D gels of AMA cellular proteins. (*A*) Fraction of an IEF gel fluorogram of AMA cell proteins labeled for 16 hr with [^{35}S]methionine (1 mCi/ml) in minimal essential medium containing unlabeled methionine at 1 mg/liter. The numbers indicating the EF-2 variants correspond to those in the 2D gel AMA human protein data base (18–20). (*B*) Coomassie brilliant blue staining of EF-2 variants from rabbit reticulocyte mixed with [^{35}S]methionine-labeled proteins from AMA cells. (*C*) Autoradiogram of the gel shown in *B*. (*D*) 2D gel immunoblot of AMA proteins (asynchronous cells) with rabbit EF-2 antibodies kindly provided by A. Minin. A small amount of [^{35}S]methionine-labeled AMA proteins was mixed with the unlabeled proteins to facilitate the identification of the spots. Immunoblotting was carried out as described by Celis *et al.* (15). Reference proteins are indicated in *A*. Molecular masses in kDa are indicated to the right.

various spots is, therefore, different from that observed when a lesser amount of protein is applied to the gel.

Interestingly, earlier studies of Bravo and Celis (21) showed that the rate of synthesis of IEF polypeptide 2714 [IEF 7 in the HeLa protein catalogue (22)] changed during mitosis in human HeLa cells. To investigate these results further, we analyzed the rate of synthesis of EF-2 proteins throughout the cell cycle of AMA cells as mitotic AMA cells can be easily obtained by mechanical detachment. Mitotic AMA cells exhibited a 35–40% decrease in the rate of protein synthesis as compared to interphase cells: this value was determined (*i*) by counting grains in autoradiograms of asynchronous cells labeled for 30 min with a mixture of 16 [^{14}C]amino acids (Fig. 2) and (*ii*) by scintillation counting of mitotic and G_1 cells labeled for 19 min with [^{35}S]methionine. Fig. 3 *A* and *B* shows the appropriate regions of representative gels of G_1 and mitotic cells, respectively, labeled for 30 min with [^{35}S]methionine. At least 80% of the cells remained in mitosis (mainly telophases) at the end of the

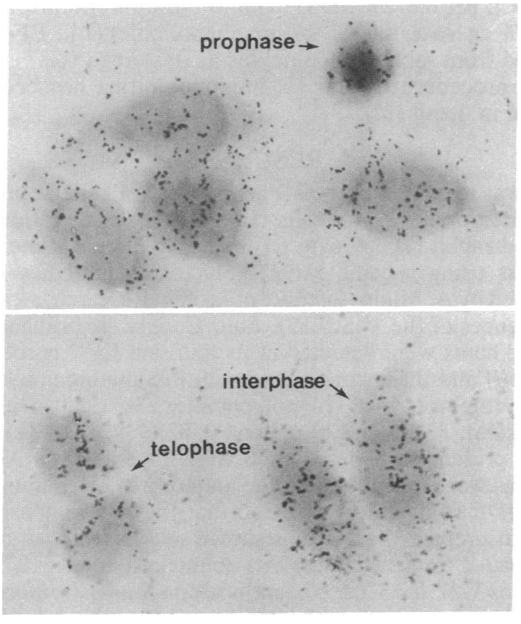

FIG. 2. Autoradiograph of asynchronous AMA cells labeled for 30 min with a mixture of 16 [^{14}C]amino acids (10 μCi/ml; Amersham, CFB 104).

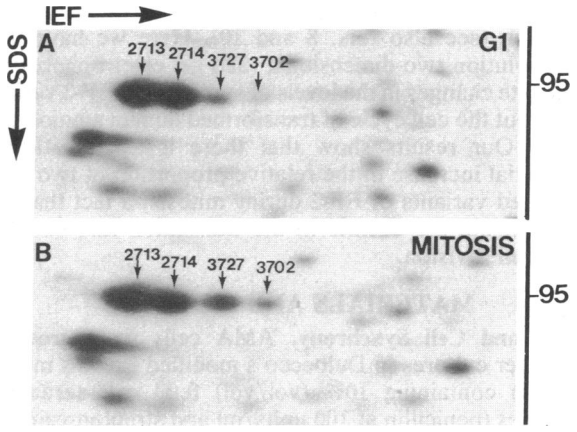

FIG. 3. Synthesis of EF-2 variants in G_1 and mitotic AMA cells. Mitotic cells obtained by shake off were labeled for 30 min with [^{35}S]methionine as described in Fig. 1. G_1 cells were labeled for the same period of time 4.5 hr after plating mitotic cells. Only the relevant area of the 2D gels is shown. Arrowheads can be used as reference to compare the various figures. Molecular mass at 95 kDa is indicated.

Table 1. Relative proportion of EF-2 variants and other proteins in G_1 and mitosis

Number in the 2D gel protein data base*	Protein	Ratio polypeptide/alkaline phosphatase		Ratio mitosis/G_1
		G_1	Mitosis	
IEF 2713	EF-2	16.7	16.4	0.98
IEF 2714	EF-2	16.0	11.4	0.71
IEF 3727	EF-2, phosphorylated variant	2.5	6.3	2.52
IEF 3702	EF-2, phosphorylated variant	1.3	2.9	2.23
IEF 7523	α-Tubulin	25.1	57.5	2.29
IEF 6610	hsx70	3.0	6.6	2.20
IEF 6407	Keratin 7	7.6	5.4	0.71
IEF 5703	Primatin, nuclear factor IV	3.1	3.6	1.16
IEF 5206	Lactate dehydrogenase (L chain)	8.4	8.3	0.99
IEF 6614	Mit-3, grp75	8.8	8.6	0.98
IEF 9218	Cyclin/PCNA	2.4	2.6	1.08
IEF 4822	Vinculin	1.5	1.8	1.20
IEF 3217	Lipocortin I	2.7	2.9	1.07
IEF 8717	grp80	3.2	3.4	1.06
IEF 7512	Mit-2, hsp58, and hsp60	12.0	13.2	1.1
IEF 6618	Alkaline phosphatase	1.0	1.0	1.0
IEF 7727	α-Actinin	8.6	8.8	1.02
IEF 9215	Tropomyosin	3.5	3.8	1.08

Mitotic AMA cells obtained by shake off were labeled for 25 min with [^{35}S]methionine. G_1 cells were labeled for 30 min, 4.5 hr after mitotic shake off. After 2D gel electrophoresis and fluorography (25), the gels were dried and the spots were cut out with the aid of an overlay x-ray film. Radioactivity in samples was measured in a LKB 1209 RackBeta liquid scintillation counter. Mitotic cells exhibited a 35–40% decline in protein synthesis. When equal amounts of trichloroacetic acid-precipitable cpm were added to the first-dimension gels, many of the quantitated spots showed similar cpm in G_1 and mitosis. The proportion of each spot relative to alkaline phosphatase (IEF 6618) (18, 20) is given for some polypeptides of known identity (see also ref. 26 for acknowledgements concerning the identification of these proteins). grp, Glucose-regulated protein; hsp or hsx, heat shock proteins; mit, mitochondrial protein; PCNA, proliferating cell nuclear antigen.
*From refs. 18 and 20.

labeling period as judged by phase-contrast microscopy. Quantitations of the four EF-2 variants as well as of proteins whose relative proportions are known to increase [α-tubulin and hsx70 (21, 23)], decrease (keratin 7) (24), or remain apparently constant during mitosis [primatin or nuclear factor IV, lactate dehydrogenase, Mit-3 or glucose-regulated protein 75 (grp75), cyclin/proliferating cell nuclear antigen (PCNA), vinculin, lipocortin I, grp80, etc.] (21, 23) are given in Table 1. The relative proportion of each polypeptide was calculated on the basis of the amount of radioactivity present in alkaline phosphatase (IEF polypeptide 6618), as the level of this polypeptide was remarkably similar both in mitosis and G_1 when the same number of cpm was applied to the gels. Clearly, the relative proportions of variants IEF polypeptides 3727 and 3702 increase dramatically during mitosis (Fig. 3 *A* and *B*) with a concomitant decrease in the level of variant IEF polypeptide 2714 (Table 1). The relative proportion of polypeptide IEF 2713 on the other hand changed only slightly during mitosis (Table 1). Similar results to those reported for G_1 cells were observed for cells at the G_1/S and S phase of the cell cycle as well as for early G_2 cells (results not shown).

2D gel analysis of asynchronous AMA cells labeled with [^{35}S]methionine for various periods of time (5 min, 30 min, or 2.5 hr; Fig. 4) showed that polypeptide IEF 2714 is the most abundant of the radioactive variants when cells are labeled for a short period of time (Fig. 4*A*, 5-min labeling). Interestingly, IEF polypeptides 3727 and 3702 could be detected in gels of cells labeled for 5 min in spite of the fact that very little IEF polypeptide 2713 was observed (Fig. 4*A*). This result suggests that IEF polypeptides 3727 and 3702 are most likely derived from polypeptide IEF 2714. Polypeptide IEF 2713 on the other hand is the major variant present in cells labeled for 2.5 hr (Fig. 4*C*) or longer (results not shown, but see Fig. 1*A*). Thus these experiments indicate that polypeptide IEF 2714 corresponds to the primary translation product and that it most likely is a precursor of the more basic variant polypeptide IEF 2713. At present, we do not know what modification

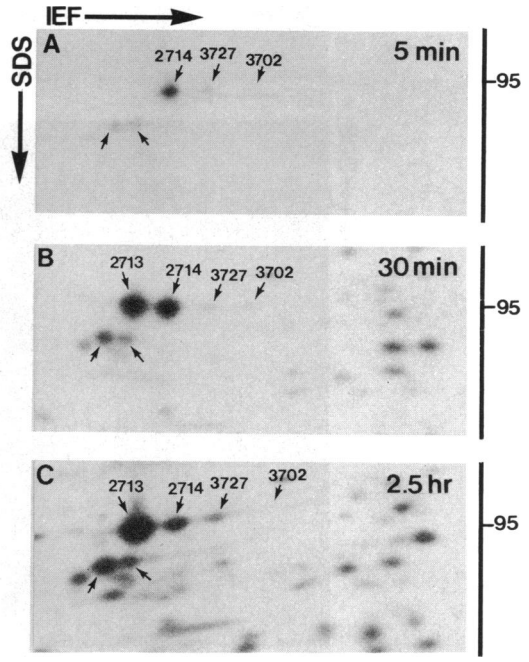

FIG. 4. Synthesis of EF-2 variants in asynchronous AMA cells labeled for various periods of time, 5 min (*A*), 30 min (*B*), and 2.5 hr (*C*). Only the relevant area of the gels is shown. Molecular mass at 95 kDa is indicated.

induces the change in mobility of polypeptide IEF 2714 toward the basic end of the gradient. It may be that polypeptide IEF 2714 corresponds to EF-2 with incompletely synthesized diphthamide (27, 28). Essentially the same four spots of EF-2 on 2D gels were observed in NIH 3T3 cells, and similar conclusions were reached (29).

Since EF-2 has been shown to be phosphorylated *in vivo* by Palfrey *et al.* (30), we analyzed the 2D gel patterns (IEF) of mitotic and G_1 AMA cells labeled metabolically with [^{32}P]orthophosphate. As shown in Fig. 5, two phosphorylated forms of EF-2 that comigrated with IEF polypeptides 3727 and 3702 (results not shown) were detected in mitotic cells (Fig. 5*A*) that were present at much reduced levels in G_1 cells labeled 4.5 hr after plating mitotic cells (Fig. 5*B*). Similar results were observed in other phases of the cell cycle (results not shown). Other proteins that are phosphorylated preferentially during mitosis correspond to keratins 7, 8, 17, and 18 (24, 31) and vimentin (24, 31), the nucleolar phosphoprotein B 23 or numatrin, and an unknown phosphoprotein of 23 kDa (Fig. 5 *A* and *B*).

DISCUSSION

By taking into consideration the above results and the fact that *in vitro* EF-2 becomes inactive after phosphorylation (7, 8, 10–12), our experiments suggest that there may be a partial inactivation of this factor during mitosis in AMA cells. This correlates with the fact that the rate of protein synthesis decreases by up to 35–40% during this phase of the cell cycle in these cells. It should be stressed that phosphorylated EF-2 is not only inactive in translation but it also inhibits translation (10). Whereas inhibition of protein synthesis during mitosis is well documented (1, 5), there is no unequivocal hypothesis as to the mechanisms underlying such inhibition. In most studies, disaggregation of polyribosomes during mitosis has been reported (1, 4, 5), and inhibition of the initiation stage has been proposed as the mechanism responsible for the regulation (1, 4, 5). In some other studies no disaggregation of polyribosomes was found (2), and in one case inhibition of polypeptide chain elongation was reported (3). These discrepancies may be explained due to differences in the cell types studied and/or to different methods used for

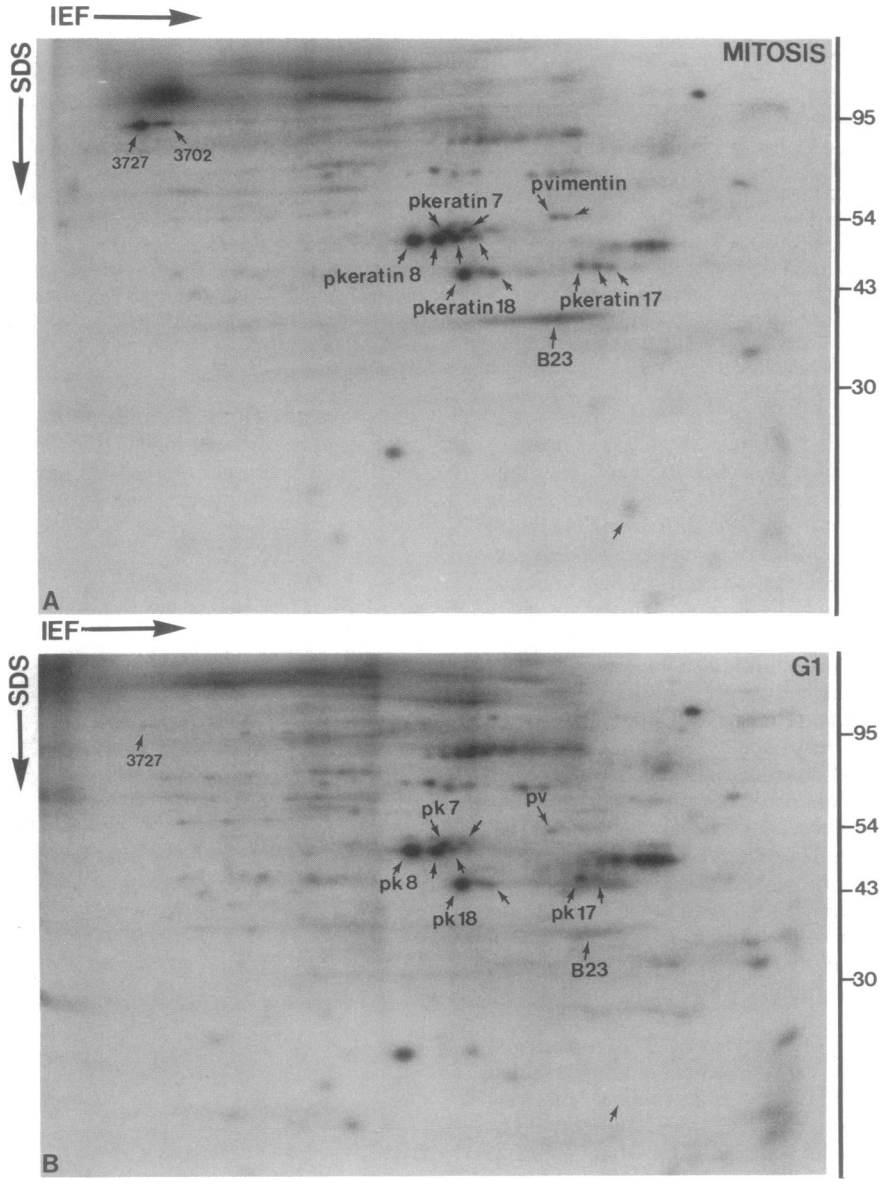

FIG. 5. 2D patterns (IEF) of [^{32}P]orthophosphate-labeled proteins from mitotic and G_1 cells labeled for 25 min with 2 mCi/ml in phosphate-free DMEM. In addition to the phosphorylated EF-2 variants, we have indicated in this figure phosphorylated keratins (pk) and vimentin (pv), which take place preferentially during mitosis (24, 31). The levels of phosphorylated nucleolar protein B23 as well as of an unknown phosphoprotein of 23 kDa also increase during this phase of the cell cycle. Molecular masses in kDa are indicated.

the preparation of mitotic cells. It is well known that dephosphorylation of eukaryotic initiation factor 4E takes place during mitosis (5) and, therefore, a decrease in the rate of protein synthesis during this phase of the cell cycle may be due to inhibition of both initiation and elongation.

In any event, the results reported here demonstrate a correlation between increased *in vivo* EF-2 phosphorylation and decrease rate of protein synthesis. The observed phenomena also provide an explanation for the role of the EF-2 kinase and lead to the suggestion of how the cell controls (at least in part) inhibition of protein synthesis in mitosis. EF-2 kinase can be activated *in vivo* by increasing the intracellular Ca^{2+} concentration (28, 30) and inactivated by increasing levels of cAMP (30). Since during mitosis there is a transient increase of Ca^{2+} (34) and a transient decrease of cAMP (35), it is likely that both of these events may induce an increase in EF-2 phosphorylation. Thus, changes in the concentration of these second messengers may inactivate EF-2 (partially) and lead to a reduced rate of protein synthesis.

The observed phenomena can also provide a clue to another intriguing fact. The synthesis of various proteins is not inhibited to the same extent during mitosis (ref. 21 and Table 1). One can ask if the changes in the degree of EF-2 phosphorylation can provide this selective inhibition of translation. Two pieces of evidence can be pointed out in this connection. (*i*) There is the experimental evidence that the efficiency of translation of some mRNAs can be modulated by EF-2 concentration. As reported by Svitkin and Agol (36), there is a translational barrier in encephalomyocarditis virus mRNA when it is translated in a fractionated cell-free system. This translation barrier can be overcome by increasing the EF-2 concentration (36). This is a clear indication that different mRNAs may need different concentrations of EF-2 for their efficient translation. (*ii*) Selective inhibition of translation of certain mRNAs can be provided by localized activation of the EF-2 kinase. The rise in the intracellular Ca^{2+} concentration during mitosis is restricted to only several defined regions of the cytoplasm (34). At the same time, the distribution of at least some types of mRNAs is not uniform throughout the cytoplasm (37). It is, therefore, tempting to speculate that the local increase in the intracellular Ca^{2+} concentration may provide local activation of the EF-2 kinase with a corresponding inhibition of translation of those types of mRNAs that are located in the same region.

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