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Multiple phosphorylation of ribosomal protein S6 during transition of quiescent 3T3 cells into early G_1, and cellular compartmentalization of the phosphate donor

(serum/two-dimensional electrophoresis/pulse–chase/metabolic stability/phosphate and ATP pools)

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ABSTRACT **At 5 min after quiescent cells are induced to enter G_1 there is a large increase in the amount of ^{32}P incorporated into 40S ribosomal protein S6. Here we show that changes in the specific activities of $^{32}P_i$ and [γ-^{32}P]ATP in stimulated as compared to quiescent cultures do not account for this large increase. Instead, we demonstrate by decreased electrophoretic mobility on two-dimensional polyacrylamide gels that this increase is due to a quantitative increase in the total amount of phosphate incorporated into S6. Furthermore, pulse–chase experiments show that the phosphate that is incorporated into S6 is metabolically stable during at least the first 60 min of induction and that the incorporation of ^{32}P into S6 responds immediately to the replacement of $^{32}P_i$ by P_i in the medium, in contrast to [γ-^{32}P]ATP which changes very slowly. Thus, the S6 phosphate donor must be a compartment separate from that of the total cellular ATP.**

Regulation of cellular proliferation in the whole animal and in tissue culture is controlled at some point in the cell cycle after mitosis and before cells have progressed far into G_1 (1, 2). Elucidation of the molecular mechanisms of this phase of the cell cycle therefore is crucial to our understanding of the biochemical processes that control cellular proliferation. One of the earliest events affected when animal cells are induced to reenter the G_1 phase of the cell cycle is a dramatic shift of monosomes to polysomes accompanied by a large increase in protein synthesis (3–5). Because protein phosphorylation/dephosphorylation reactions may be common mediators of cellular regulatory signals (6–8) and because ribosomal proteins are known to be phosphorylated both *in vitro* and *in vivo* (for reviews, see refs. 9 and 10), we have explored the possibility that phosphorylation of specific ribosomal proteins may be involved in the transition of quiescent cultures into the G_1 phase of the cell cycle.

It has been reported (11) that at 5 min after induction of secondary chicken embryo fibroblasts with serum, insulin, or insulin-like growth factor, there is a large increase in the amount of ^{32}P incorporated into 40S ribosomal protein S6. In the study described here, using 3T3 fibroblasts we found that the increase in incorporation of ^{32}P was due to a quantitative increase in the phosphorylation of S6. This was carried out by comparison of the specific activities of ^{32}P in P_i and ATP γ-phosphate pools and by examination of the change in S6 mobility on two-dimensional polyacrylamide gels. We have also examined the metabolic stability of the phosphate in S6 during the stimulation. In so doing, we have discovered evidence for the presence of a compartment for the phosphate donor pool, distinct from the total cellular ATP pool. Finally, the implications of S6 phosphorylation in relation to other known cellular events taking place during early G_1 phase of the growth cycle are discussed.

MATERIALS AND METHODS

Cell Cultures. Swiss mouse 3T3 fibroblasts were seeded at 5×10^5 cells per 80-mm tissue culture plate in 10 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum, as described (12). After 72 hr, cultures were re-fed with the same medium supplemented with 0.03 μM biotin, 0.05 μM vitamin B_{12}, 140 μM aspartic acid, 350 μM glutamic acid, 50 μM glutathione, and 60 μM hypoxanthine (13). Cultures were judged to be quiescent at 7 days after seeding when no mitotic cells were observed.

Preparation of Ribosomal Subunits. Lysates from cell cultures, total cellular ribosomes, and ribosomal subunits were prepared as described (11, 12) except that ribosomal subunits were separated on 4-ml isokinetic gradients in buffer B (11). The gradients at the top and in the reservoir contained 5.1% and 30.2% sucrose (wt/vol), respectively, and the constant-volume mixing chamber for six gradients was 26.1 ml. Gradients were centrifuged at 18°C for 48 min at 407,000 $\times$ *g* max (Beckman L5-65 centrifuge, SW 60 rotor).

Polyacrylamide Gel Electrophoresis. Preparation of protein samples, electrophoresis, and autoradiography were carried out as described (11) except that all sodium dodecyl sulfate/polyacrylamide gel electrophoresis solutions were made 6 M in urea (14). The amount of [^{32}P]phosphorus incorporated into S6 was determined by scanning autoradiograms with a Vitatron TDL-100 densitometer (Dieren, Holland) and comparing the amount present with known standards. Fluorography was carried out as described by Bonner and Laskey (15).

Determination of P_i and ATP-γ-Phosphate Specific Activities. Cells were washed twice with cold 0.15 M NaCl/20 mM Hepes, pH 7.0, extracted for 15 min with 4 ml of cold 2 M formic acid and then with 2 ml of additional formic acid. The extracts were pooled and evaporated to dryness, and the residues were resuspended in 0.2 ml of water. A portion of each sample was then used, as described below, for determining the P_i and ATP-γ-P specific activities. Extraction of total P_i as phosphomolybdate into an organic hyperphase was carried out as described (16), except for the omission of silicotungstate. The amount of total P_i present, determined at 820 nm, and the amount of $^{32}P_i$, counted by Cerenkov radiation, were measured from the same sample. Total ATP was determined by minor modification of the bioluminescence assay (17). The 1-ml reaction mixture contained 20 mM Tris·HCl at pH 7.4 and 0.1 mM $MgCl_2$ and the reaction was initiated by the addition of 5 μl of luciferin/luciferase (Boehringer; 20 mg/ml in water). The luminescence was measured in a Beckman β-mate manual scintillation counter.

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Table 1. ^{32}P specific activities in quiescent and serum-stimulated cultures

	Specific activity, cpm/mol		Ratio, stimulated/ quiescent
	Quiescent	Stimulated	
S6	0.31	8.62	27.75
ATP-γ-phosphate	1.97	4.17	2.11
P_i	12.60	12.80	1.02

Quiescent cultures of 3T3 cells, were treated with 0.15 M NaCl (quiescent) or 15% (by volume) fetal calf serum (stimulated) in the presence of $^{32}PO_4$ (0.11 mCi/ml; Amersham; 1 Ci = 3.7×10^{10} becquerels). For calculation of the specific activity of [^{32}P]S6 an 80S monosome molecular weight of 4.5×10^6 (18), an extinction coefficient of 11.3 A_{260}·cm^3·mg^{-1} of 80S monosome, and 1 mol of S6 per mole of 80S monosome (19) were assumed. Each value represents the mean of two separate determinations.

To determine the amount of [γ-^{32}P]ATP, advantage was taken of the exchange reaction between the γ-phosphate of nucleoside triphosphates in the presence of nucleoside diphosphate kinase (Boehringer):

$$[\gamma\text{-}^{32}P]NTP + GTP \overset{\text{kinase}}{\rightleftharpoons} NTP + [\gamma\text{-}^{32}P]GTP.$$

At equilibrium the γ-phosphates reach constant specific activity. Therefore, in the presence of a large excess of GTP (GTP radioactivity was less than 10% of that of ATP, and that of CTP and UTP was much less than that of GTP), virtually all the γ-phosphate radioactivity is shifted into GTP. This was verified in separate experiments with known amounts of [γ-^{32}P]ATP. The 25-μl reaction mixture contained 100 nmol of GTP, 4 mM $MgCl_2$, and 20 mM triethanolamine·HCl at pH 7.8. After 5 min at 37°C the samples were applied to sheets of polyethyleneiminocellulose (Machrey–Nagel) and subjected to thin-layer chromatography. Chromatography was carried out in 1.5 M LiCl in the first dimension and in 0.75 M KPO_4 at pH 3.4 in the second dimension; in both dimensions, solvent fronts reached the top of the plates. Prior to and after each step of chromatography, chromatograms were washed for 15 min in methanol and then allowed to dry for 5 min at room temperature. After chromatography each chromatogram was subjected to autoradiography, the GTP and ATP spots (identified with known markers) were cut out, and the radioactivity was measured as Cerenkov radiation. The amount of [γ-^{32}P]ATP was then determined from the difference between identical samples treated in the presence or absence of nucleotide diphosphate kinase.

RESULTS

When quiescent cultures of 3T3 fibroblasts were stimulated for 30 min in the presence of serum, there was a 28-fold increase in the amount of ^{32}P incorporated into 40S ribosomal protein S6 compared to control cultures (Table 1). Because the stimulation of quiescent cultures by serum leads to a large increase in phosphate transport (20–23) and in the cellular ATP content (24), one possibility is that the increase in S6 radioactivity was due to an increase in the specific acitivty of the intracellular phosphate pools. However, the specific activity of [γ-^{32}P]ATP in stimulated cultures was little more than twice that of control cultures. The specific activity of $^{32}P_i$ was almost identical in stimulated and control cultures. Thus, the total increase in incorporation of ^{32}P into S6 cannot be simply attributed to a change in the specific activity of the [γ-^{32}P]ATP.

If this increase is not due to changes in the specific activity of the S6 phosphate donor, this strongly argues, as previously suggested (11), that the increase is due instead to the amount of phosphate incorporated into S6. The degree of phosphorylation of S6 can be measured by its mobility on two-dimensional polyacrylamide gels (25–27). Thus, 40S ribosomal proteins derived from stimulated and control cultures prelabeled with [^{35}S]methionine were separated by two-dimensional polyacrylamide gel electrophoresis and analyzed by fluorautography. S6 from unstimulated cultures migrated as an elongated spot [which we now know to be two spots (unpublished data)] located on a diagonal line with 40S ribosomal proteins S2 and S4 (Fig. 1*A*). Thirty minutes after serum stimulation the protein was almost totally relocated in two new positions, the major species, S6c, migrating directly below S2 and the minor species, S6b, migrating directly below S4 (Fig. 1*B*). In addition, there were

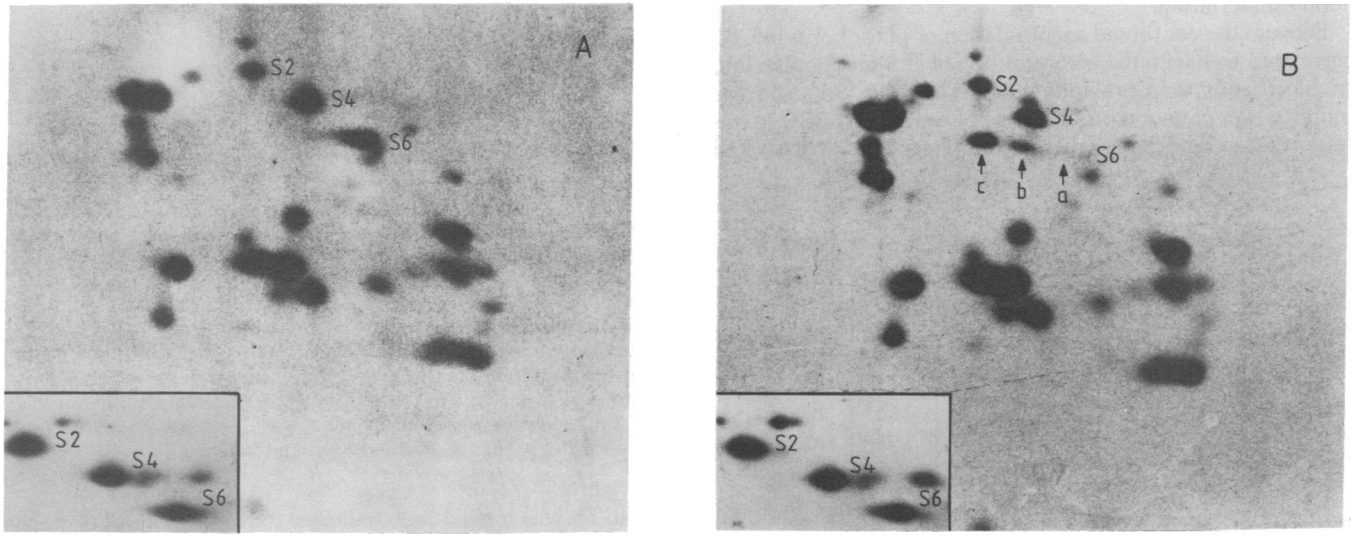

FIG. 1. Two-dimensional polyacrylamide gel electrophoresis from cultures treated with NaCl (*A*) or serum (*B*), to measure extent of S6 phosphorylation. When the media were changed, 3 days after initial seedings, [^{35}S]methionine (10 μCi/ml) was added to each culture. After 4 days, cultures were judged to be quiescent and were treated for 30 min with either NaCl or serum as described in Table 1. Ribosomes were then harvested, subunits were separated, and the proteins were analyzed by two-dimensional polyacrylamide gel electrophoresis. An aliquot of 40S subunits prepared as above in buffer A (20) was incubated with alkaline phosphatase (EC 3.1.3.1 from calf intestinal mucosa, a gift from P. Portmann and J. F. Comment (Université de Fribourg, Switzerland), 0.5 μg per μg of 40S ribosomal protein, for 1 hr at 37°C and the proteins were analyzed. (*Insets*) After alkaline phosphatase treatment of 40S proteins.

Table 2. ^{35}S present in each derivative of S6

Derivative	% of total	
	Quiescent	Serum stimulated
Native	80	6
S6a	10	2
S6b	6	20
S6c	4	72

The two-dimensional polyacrylamide gels in Fig. 1 were aligned with the corresponding autoradiograms and the S6 derivatives were cut out, eluted, and assayed for radioactivity as described by Ward *et al.* (28).

detectable amounts of S6 migrating in the same position as native S6 and S6a. To quantitate the absolute amount of S6 present in each species, the ^{35}S radioactivity in each derivative was determined (Table 2). In quiescent cultures, 90% of S6 was in positions S6 and S6a. However, after serum induction, >80% shifted to positions S6b and S6c. To ensure that the shift in electrophoretic mobility was due to phosphorylation, small aliquots of 40S proteins from serum-stimulated and control cultures were treated with alkaline phosphatase and the proteins were examined as described above (Fig. 1 *insets*). S6 derived from serum-stimulated cultures then migrated in a single position identical to that of resting cultures. Thus, the increase in the amount of ^{32}P incorporated into S6 (Table 1) is largely due to the quantitative increase in the amount of phosphate incorporated into S6.

Previously, it had been found that, as secondary chicken embryo fibroblasts progress further into G_1, the ability to incorporate radioactive phosphorus into S6 diminishes (11). Similar results were obtained here with 3T3 cells (Fig. 2*A*). From these results (11) and an earlier report (29) that the half-life of ribosomal protein phosphorylation was of the order of 20 min, we suggested that S6 was only transiently phosphorylated during this time. However, when 3T3 cells were induced at 0 time in the continuous presence of ^{32}P, the amount of ^{32}P incorporated into S6 continued to increase (Fig. 2*B*). Thus, the data from Fig. 2 imply that there was a net accumulation of phosphate in S6 during this time and no apparent dephosphorylation.

Because the continued accumulation of phosphorus into S6 correlated well with the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitate material (Fig. 3*A*) and the mobilization of monosomes into polysomes (Fig. 3*B*), it was important to determine whether the phosphate incorporated

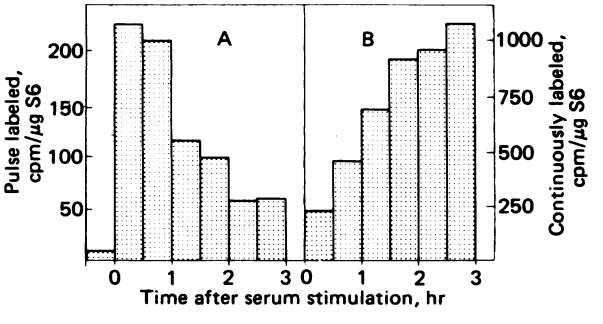

FIG. 2. Ability of S6 to be phosphorylated as a function of time after serum induction. (*A*) Serum, 15% (vol/vol), was added to quiescent cultures at 0 time and $^{32}PO_4$, 0.11 mCi, was added for 30-min pulses beginning 30 min prior to the addition of serum. At the end of each $^{32}PO_4$ pulse, cultures were harvested and S6 was extracted and measured. (*B*) Same as A, except serum and $^{32}PO_4$ were added together at 0 time and cells were harvested every successive 30 min. Each bar represents the mean of two separate determinations.

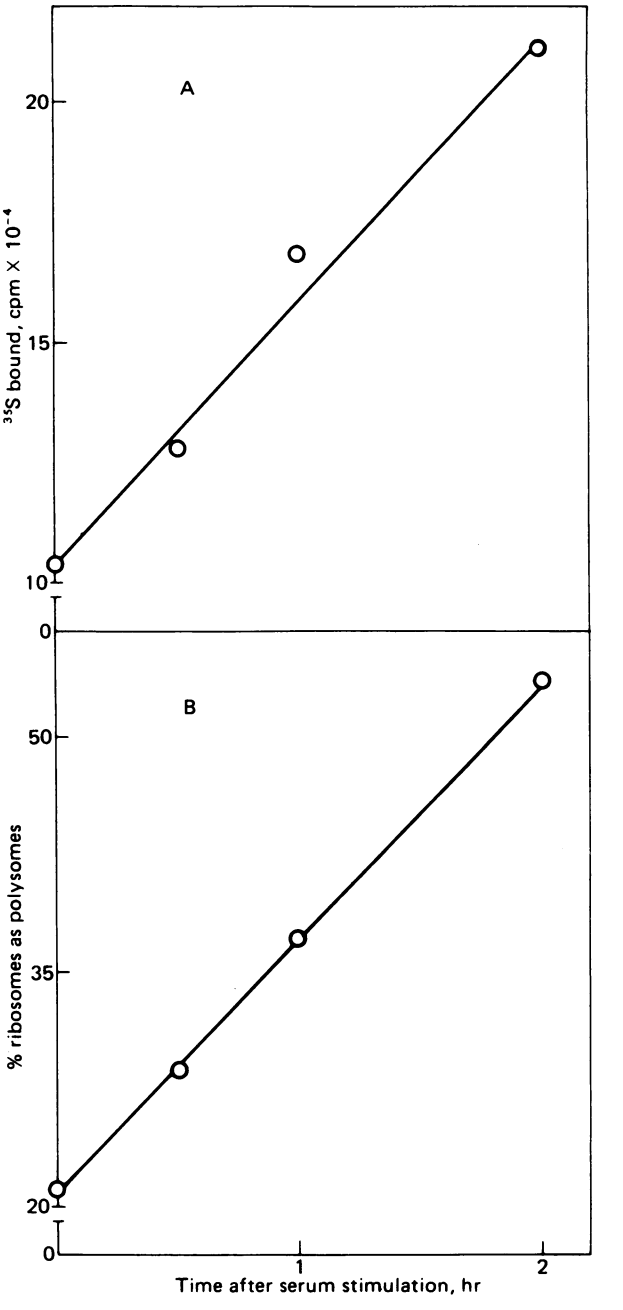

FIG. 3. Incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material and percentage of total ribosomes as polysomes at various times after addition of serum. (*A*) Cultures were pulse-labeled for 20 min with [^{35}S]methionine (1.3 mCi/ml). Lysates were prepared as described (12) and made 10% in trichloroacetic acid; the precipitates were collected on GF/C filters, dried, and assayed for radioactivity in 5 ml of Bray's solution (12). Each time point represents the midpoint of the pulse. (*B*) Cultures were extracted and the lysates were analyzed on isokinetic sucrose gradients (12). Those areas of the recording representing cellular ribosomes (subunits, monosomes, and polysomes) were cut out and weighed.

into S6 was indeed metabolically stable. This has been confirmed directly with pulse–chase experiments. Quiescent 3T3 cells were labeled with ^{32}P during the first 30 min after stimulation and the intracellular specific activities of ^{32}P in S6, ATP, and P_i were determined at successive times after replacement with nonradioactive medium (Fig. 4). The rate of accumulation of ^{32}P in S6 immediately became zero and remained at this level throughout the experiment. However, the [γ-^{32}P]ATP de-

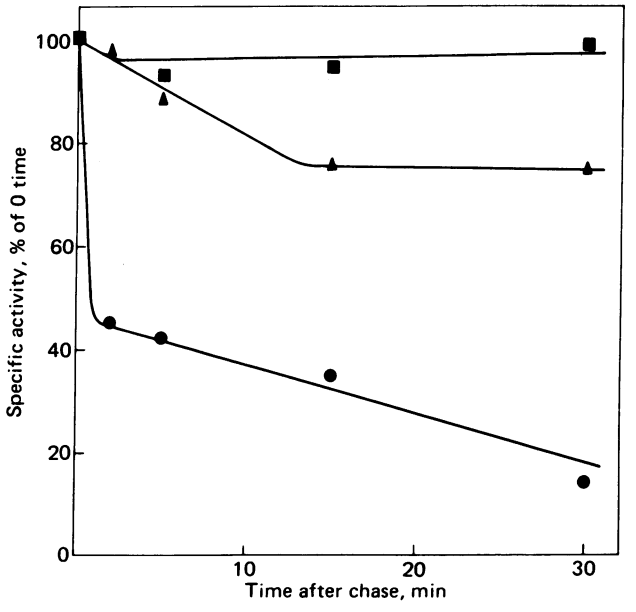

FIG. 4. Specific activities of [^{32}P]S6 (■), [γ-^{32}P]ATP (▲), and $^{32}P_i$ (●) at various times after removal of $^{32}PO_4$ from the medium. Quiescent cultures were stimulated as in Table 1. After 30 min, cultures were rinsed twice with 5 ml each of conditioning medium containing 15% (vol/vol) serum and incubated for the times indicated with an additional 5 ml of the same medium. Each point represents the mean of two separate determinations.

creased only 25%, exchanging slowly with the P_i in the medium. The intracellular $^{32}P_i$ pool, on the other hand, showed an initial rapid decline followed by a slower exchange phase. Thus, the S6 phosphate during this time of the cell cycle was metabolically stable and, because there was no further increase in the amount of [^{32}P]S6 during the chase, the phosphate donor must have been a separate compartment from the total intracellular pool of ATP.

DISCUSSION

The results in Fig. 4 show that the fraction of ATP that is the S6 phosphate donor, unlike the total cellular ATP pool, is in rapid equilibrium with the medium. The finding that total ATP exchanges relatively slowly with the medium is in good agreement with a comparable study carried out by Weber and Edlin (30). However, in contrast to their findings we show that a large portion of the P_i pool comes into rapid equilibrium with the medium (Fig. 4 and unpublished data). The presence of such a pool, which may not have been detected by Weber and Edlin (30) for technical reasons, gives additional support for the existence of a separate S6 phosphate donor pool that is also in rapid equilibrium with the medium. Furthermore, our findings that the specific activities of $^{32}P_i$ in quiescent and serum-stimulated cultures are almost identical are not in conflict with earlier reports showing that there are large increases in ^{32}P transport after serum stimulation (20–23). These authors measured the uptake of phosphate into acid-soluble pools rather than strictly P_i, as we measure here. Thus, because P_i was in rapid equilibrium, its specific activity will not have changed although the amount of ^{32}P incorporated into acid-soluble pools, such as ATP (Table 1), will continue to increase.

Evidence for the intracellular compartmentalization of ATP pools has come from several other laboratories. Rapaport and Zamecnik (31) have recently shown that adenosine, but not adenine or hypoxanthine, is preferentially incorporated into a separate intracellular ATP pool. Grummt and Grummt (32) have argued that a special compartment may be involved in the control of rRNA synthesis. Finally, in another context, it has been shown that mitochondria contain an ATP pool that is metabolically separated from that of the remaining cell (33). In this regard it may be interesting to determine whether the endoplasmic reticulum contains such a pool because it is known that, as 3T3 cells reach quiescence, there is a large increase in the number of membrane-bound ribosomes (34).

With regard to pools it has been suggested (9, 10) that stimulation of intracellular cyclic AMP may lead to the activation of a specific S6 phosphokinase. However, in serum-stimulated 3T3 cells, cyclic AMP levels are greatly decreased (35–38). On the other hand, cyclic GMP levels have been reported to be increasing (36, 37) and this has led others (27) to suggest that this compound may be the S6-phosphokinase activator. But again there is a great deal of controversy concerning whether cyclic GMP levels are increasing or decreasing during this time (38, 39). We also cannot exclude the possibility that, even though total intracellular pools of cyclic AMP and cyclic GMP may be decreasing, there may be a compartment that is increasing. Furthermore, there is more than one S6-phosphate derivative (Fig. 1*B*) and it may be that each is phosphorylated by a unique kinase, similar to kinases that have been described for glycogen synthetase (7). Thus, not only is there no clear candidate for a phosphokinase activator but also there could be more than one.

Even though the ATP pool that acts as the S6 phosphate donor is in rapid equilibrium with the medium, it is clear from the results in Fig. 1 and Table 2 that the increase in phosphorylation is due to a quantitative increase in the amount of phosphate incorporated into S6. In contrast to these findings, under similar conditions Leader *et al.* (26) found no measurable change in S6 phosphorylation. These differences may be explained by the extent of cellular arrest. Indeed, Lastik *et al.* (27) have observed that HeLa cells grown to different densities and resuspended for 2 hr in fresh medium exhibit differential levels of S6 phosphorylation. Further support for increased S6 phosphorylation during G_1 also comes from studies in regenerating rat liver. Cells induced to proliferate after partial hepatectomy have been shown to contain a large amount of phosphorylated S6 (25). That we report a more rapid and extensive effect than these authors (25–27) is most likely accounted for by the higher degree of cell synchrony as cells enter G_1.

That the apparent rate of phosphorylation decreased as cells progressed through G_1 (Fig. 2*A*), although the accumulation continued linearly for up to 2 hr, may be due to pool effects or turnover at later times. We have not explored this beyond 1 hr. However, we have observed that S6 is rapidly dephosphorylated when serum is removed from the medium of either quiescent or G_1 cells.

The almost complete phosphorylation of S6 immediately after induction (Fig. 1 and Table 2) argues that it is not involved in ribosomal maturation [as has been suggested elsewhere (25, 26)] because there is no net accumulation of new ribosomal subunits for 8 hr (5, 39) nor is a metabolically stable S6 consistent with involvement in a catalytic step of protein synthesis. A more interesting possibility is that the phosphorylation of S6 could fall into a category of events described by Lodish (40, 41) that may lead to changes in the affinity of 40S subunits for cellular mRNA and thus alter the pattern of translation. It has been reported that S6 (along with S8) has been crosslinked to the synthetic mRNA poly(U) (42). Furthermore, it has been shown that mRNAs from quiescent and growing cells are similar (43). However, quiescent cultures contain a great deal of nontranslated mRNA (4, 44) and the activity of many enzymes is changing during G_1 (45). Thus, phosphorylation of S6 could

play a role in the regulation of expression of mRNA during this phase of the cell cycle.

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