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Transcriptional and translational control of cytoplasmic proteins after serum stimulation of quiescent Swiss 3T3 cells

(lag phase/pulse-labeling/two-dimensional gels/new proteins)

GEORGE THOMAS, GARY THOMAS, AND HEIDE LUTHER

Friedrich Miescher-Institut, P.O.B. 273, CH-4002 Basel, Switzerland

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ABSTRACT **The synthesis of cytoplasmic proteins from quiescent and serum-stimulated Swiss 3T3 cells was compared by two-dimensional polyacrylamide gel electrophoresis. Four new proteins of M_rs 26,000, 28,000, 45,000, and 47,000 designated N26, N28, N45, and N47, which were not detectable in quiescent cells, appeared 60 min after addition of serum. During the same period, the amount of [^{35}S]methionine incorporated into 10 proteins present in quiescent cells, ranging in M_r from 23,000 to 98,000 and designated Q23–98, increased up to 6-fold, whereas the amount incorporated into three other proteins decreased by a factor of ≈2. Of the new proteins, N26 was no longer detectable, and the amount of [^{35}S]methionine incorporated into N47 was significantly reduced by 150 min. During this same time, a fifth new protein, N56, appeared, and there was a large increase in the amount of radioactivity incorporated into another protein, Q121. The increases in nine of the proteins were either strongly or completely inhibited by actinomycin D, arguing that the expression of these proteins was under transcriptional control. In contrast, the increases in seven other proteins were unaffected by actinomycin D, suggesting that their expression was under translational control. These proteins will serve as useful markers for determining how cells progress through early lag phase.**

When quiescent animal cells in culture are stimulated to proliferate by serum or individual growth factors, a number of complex biological processes are activated (1–3). To understand how proliferation is regulated, it will be necessary to determine how each process is controlled, how each is integrated one with another, and how the activation of these processes affects the initiation of DNA synthesis and cell division. Protein synthesis plays a central role. It is required throughout lag phase (time from addition of serum or growth factors until cells enter S phase) for the initiation of DNA synthesis (4), and during S and G_2 phases for mitosis and cell division (5). We have been studying the mechanisms by which protein synthesis may be activated in quiescent Swiss mouse 3T3 cells during early lag phase (6–8) and, more recently, how activation affects the pattern of mRNA expression.

Sixty minutes after the addition of serum to quiescent 3T3 cells, the rate of protein synthesis is increased 2- to 3-fold (6–8). The increase in protein synthesis is accompanied by the attachment of preexisting nonpolysomal mRNA and newly transcribed mRNA to inactive 80S monosomes and their shift into actively translating polysomes (9–11). Little is known about the mRNA sequences of either class and whether they differ from those that are translated in quiescent cells. It has been shown by cross-hybridizing cDNA and mRNA from resting and exponentially growing mouse 3T6 cells that the majority of the total cytoplasmic mRNA sequences are identical (12). However, 3% of the sequences from either population do not cross-hybridize, suggesting that gene expression is altered as a function of the growth state of the cell. This argument is in agreement with earlier studies of Salas and Green (13) and Fox and Pardee (14), who found quantitative differences in DNA binding proteins as a function of cell growth. Additional support comes from the more recent studies of Gates and Friedkin (15) and Riddle *et al.* (16), who have presented evidence for quantitative changes in the synthesis of one or more proteins after serum stimulation of 3T3 cells.

To understand how the expression of mRNA is regulated during early lag phase, a comprehensive map of protein changes after serum stimulation of quiescent 3T3 cells is required. Next, it must be determined whether these protein changes are controlled by alterations in mRNA expression at the translational or transcriptional level. We describe here a detailed application of two-dimensional polyacrylamide gels (17) to this problem. The results show that, after addition of serum to quiescent 3T3 cells, there are numerous changes in the pattern of protein synthesis and that these changes reflect apparent alterations in the expression of mRNA at both the translational and transcriptional level.

MATERIALS AND METHODS

Cell Culture and Labeling. Swiss mouse 3T3 cells were seeded at 6×10^4 cells per 35-mm tissue culture plate in 2 ml of DME medium (Dulbecco's modified Eagle's medium) containing 10% (vol/vol) fetal calf serum as described (18). After 72 hr the medium was replaced with DME medium S (DME medium supplemented with 0.03 μM biotin, 0.65 μM vitamin B-12, 140 μM aspartic acid, 350 μM glutamic acid, 50 μM glutathione, 60 μM hypoxanthine, and 6% fetal calf serum) (19). No mitoses were observed 7–8 days after seeding, and the cells were judged quiescent. To pulse-label cytoplasmic proteins to high specific activity with [^{35}S]methionine, it was first necessary to determine the lowest concentration of methioine in DME medium S at which cells could be maximally stimulated to initiate DNA synthesis. Therefore, the medium was removed from six parallel cultures of quiescent cells, each culture was then rinsed twice with 2 ml of phosphate-buffered saline, followed by incubation with 2 ml of DME medium S containing 20% dialyzed fetal calf serum, 5.5 μCi (1 Ci = 3.7×10^{10} becquerels) of [*methyl*-^{3}H]thymidine, and either no methionine or methionine at 1%, 10%, 50%, or 100% (30 μg/ml) of the normal concentration of methionine in DME Medium S. After 22 hr, labeled nuclei were assayed as a measure of the number of cells that initiated DNA synthesis (19). It was found that 5% methionine (1.5 μg/ml) was as efficient as 100% methionine for initiating DNA synthesis (80–90% of the cells entered S phase) (data not shown). Therefore, 5% methionine was used in all of

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Abbreviation: DME medium, Dulbecco's modified Eagle's medium.

the labeling experiments described here with serum-stimulated cultures. To pulse-label cultures, the volume of medium in each plate was lowered to 0.5 ml, and 50 μl of [^{35}S]methionine (250 μCi) in the same medium was added to each plate for 20 min. To chase the [^{35}S]methionine label from nascent into completed proteins, the cells were rinsed twice with 2 ml of DME medium S (containing 20% serum and 30 μg of methionine per ml), then 2 ml of this same medium was added to each plate, and the incubation was continued for another 20 min. Cells were then harvested. Quiescent cells were labeled as serum-stimulated cells except in conditioned medium (the medium in which they came to arrest). This was done to minimize the possibility of generating artifacts through altering the constituents of the medium. Quiescent cells also were labeled for 60 min rather than 20 min because the rate of protein synthesis was lower by a factor of 2–3 than the rate in serum-stimulated cells and because they were labeled in conditioned medium, which contains a high concentration of methionine. The pattern of protein synthesis was identical whether quiescent cells were labeled for 20, 60, or 240 min (data not shown). The chase was carried out as described for serum-stimulated cells, except with conditioned medium. To label cells to high specific activity with [^{3}H]isoleucine, it was found (in the same manner as described for methionine) that as many cells could be stimulated to initiate DNA synthesis with 1% as with 100% (105 μg/ml) isoleucine (data not shown). Thus, for labeling cells with [^{3}H]isoleucine, cultures were first washed twice with phosphate-buffered saline, then incubated in 0.5 ml of DME medium S containing 1.05 μg of isoleucine per ml, 20% dialyzed fetal calf serum, and 100 μl of [^{3}H]isoleucine (100 μCi) for 2 hr.

Extraction and Preparation of Cytoplasmic Proteins. Total cytoplasmic proteins were extracted by using the double-detergent method described for extracting cytoplasmic ribosomes (18). After removing nuclei by low-speed centrifugation, the supernatant was treated with 50 μg of ribonuclease A (Boehringer Mannheim) for 5 min at 37°C. To determine the amount of radioactively labeled protein present in each sample, 1 μl was treated and the radioactivity was assayed as described (7). At this point either the samples were frozen in liquid nitrogen and stored at −70°C or an aliquot containing 1×10^6 cpm was removed from each sample, adjusted to the same protein concentration as the other samples with unlabeled cytoplasmic protein, and mixed with 5 vol of −20°C acetone. The precipitate, which forms at −20°C in approximately 20 min, was pelleted at 0°C for 5 min at 4000 g_{max} (Sorvall RC2-B centrifuge, HB-4 rotor). The protein pellet was rinsed twice with acetone and dissolved in 50 μl of fresh first-dimensional-gel sample buffer and electrophoresis was carried out.

Two-Dimensional Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was essentially as described by O'Farrell *et al.* (17). Nonequilibrium pH-gradient electrophoresis in the first dimension was for 3.5 hr at 500 V. The second dimension was a 12.5% (wt/vol) NaDodSO$_4$/polyacrylamide gel containing 0.1% *N,N'*-methylenebisacrylamide, and electrophoresis was for 12 hr at 90V. The gels were then placed in EN^{3}HANCE (New England Nuclear) containing 1% glycerol for 1 hr, washed twice for 15 min in 20% (vol/vol) methanol/1% glycerol, dried, and analyzed by fluorography (20, 21).

RESULTS

Analysis of Cytoplasmic Proteins by Nonequilibrium pH-Gradient Electrophoresis/Two-Dimensional Gel Electrophoresis. To determine whether cytoplasmic proteins synthesized in quiescent cells were the same or different from those synthesized in cells stimulated with serum, both cultures were pulse-labeled with [^{35}S]methionine, and the radioactively labeled cytoplasmic proteins were analyzed by nonequilibrium pH-gradient electrophoresis/two-dimensional gel electrophoresis (Fig. 1). The most notable difference in the pattern of translation after serum-stimulation (Fig. 1*B*) was the *de novo* ap-

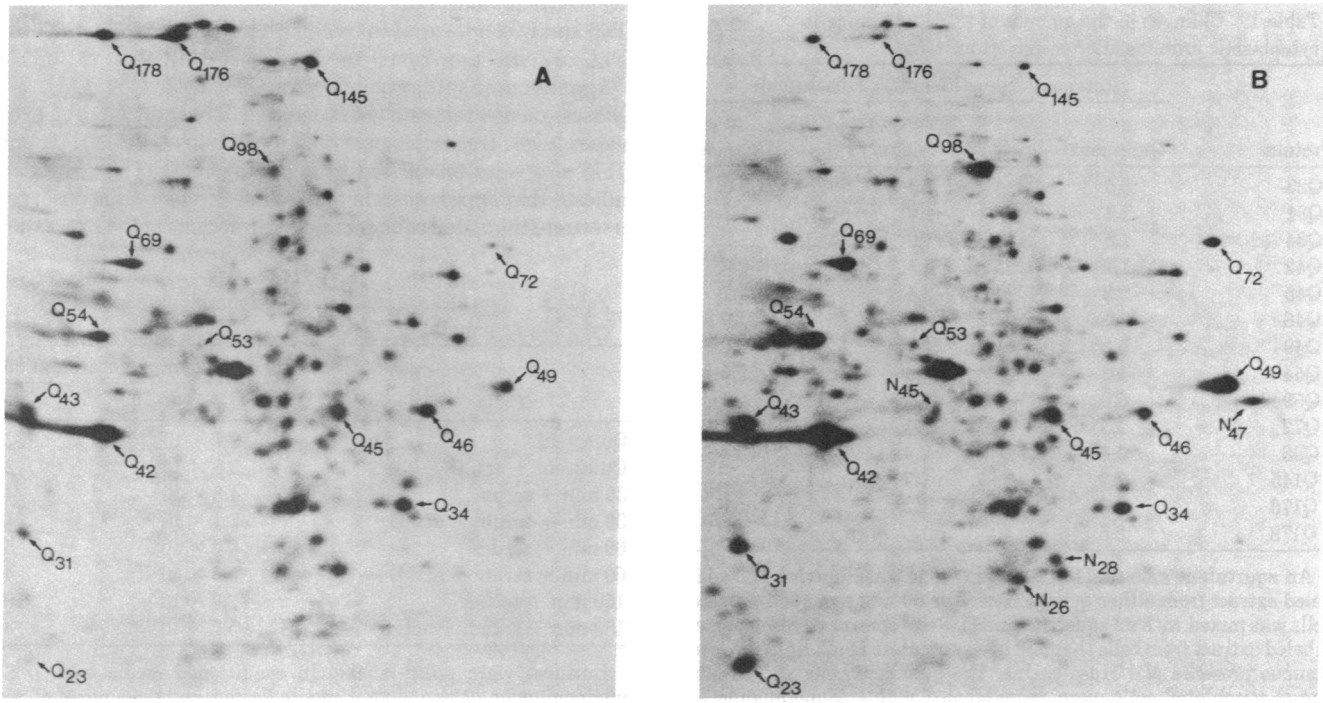

FIG. 1. Two-dimensional fluorograms of [^{35}S]methionine-labeled cytoplasmic proteins from quiescent cells (*A*) and from 60-min serum-stimulated cells (*B*). Parallel cultures of quiescent and 60-min serum-stimulated cultures were pulse-labeled with 250 μCi of [^{35}S]methionine. Quiescent cells were pulse-labeled for 1 hr and 60-min serum-stimulated cells were pulse-labeled in the 50- to 70-min interval after the change of the medium. An equivalent amount (1×10^6 cpm) of [^{35}S]methionine-labeled cytoplasmic protein was applied to each gel.

pearance of cytoplasmic proteins of M_rs 26,000, 28,000, 45,000, and 47,000 (designated N26, N28, N45, and N47). Their synthesis was not detected in quiescent cells (Fig. 1*A*). In addition, of the proteins that were synthesized in quiescent and serum-stimulated cells, four of M_rs 23,000, 49,000, 72,000, and 98,000 (designated Q23, Q49, Q72, and Q98) increased significantly as a fraction of the total labeled protein applied to the gel. Others, such as Q31, Q42 (which coelectrophoresed with actin; data not shown) Q43, Q53, Q54, and Q69, increased to a lesser extent. During this same period, the amount of three proteins (Q145, Q176, and Q178) decreased. Finally, the majority of the proteins, such as Q34, Q45, and Q46, appeared little changed.

Quantitative Analysis of Changes in Cytoplasmic Proteins. As others have done (17, 22–24), we have observed that the ability of proteins to enter the first-dimensional gel can be influenced by a number of factors. To ensure that the differences observed here were not generated by any of these factors, we performed a double-label experiment. A cell lysate from cultures that had been stimulated for 2 hr in the presence of [^{3}H]isoleucine was mixed with an equal amount (in radioactivity) of [^{35}S]methionine-labeled proteins from either quiescent or 60-min-stimulated cultures. Samples were then adjusted to the same protein concentration, extracted, and separated on two-dimensional polyacrylamide gels as described (Fig. 1). The quiescent proteins were then located on the gel by overlaying it with the fluorogram, cut out, and the ratio of [^{35}S]methionine/[^{3}H]isoleucine was determined for each. The ratios for stimulated cells were then divided by the ratios for quiescent cells. The results are in Table 1. For proteins Q23, Q49, Q72, and Q98, the quotients increased by more than 3.5-fold during this time, whereas those for Q31, Q42, Q54, and Q69 increased about 2-fold, and proteins Q34, Q45, and Q46 were little changed. Furthermore, proteins Q145, Q176, and Q178 decreased by a factor of 2. Thus, the alterations observed in the pattern of translation in Fig. 1 were not generated by selective loss of specific proteins.

Table 1. Changes in the amount of [^{35}S]methionine in cytoplasmic proteins after serum stimulation

	Ratio of [^{35}S]methionine/[^{3}H]isoleucine		
Protein	Quiescent	Serum-stimulated	Change
Q23	0.5	2.5	5.0
Q31	2.4	4.0	1.7
Q34	1.5	1.8	1.2
Q42	1.2	3.1	2.6
Q45	2.9	3.4	1.2
Q46	4.1	3.9	1.0
Q49	0.3	1.7	5.7
Q54	1.3	3.3	2.5
Q69	0.9	1.9	2.1
Q72	1.3	5.4	4.2
Q98	0.8	2.9	3.6
Q145	2.7	1.7	0.6
Q176	2.6	1.2	0.5
Q178	2.5	1.5	0.6

An equivalent amount (1×10^6 cpm) of [^{35}S]methionine pulse-labeled extract from either quiescent cells or 60-min serum-stimulated cells was mixed with an equal amount (1×10^6 cpm) of [^{3}H]isoleucine-labeled extract from cells that had been stimulated for 2 hr in the continuous presence of [^{3}H]isoleucine. The quiescent cells and 60-min serum-stimulated cells were pulse-labeled with [^{35}S]methionine as described in Fig. 1. After two-dimensional polyacrylamide gel electrophoresis, individual proteins were located by fluorography and eluted from the gel, and the amount of [^{35}S]methionine and [^{3}H]isoleucine present in each spot was determined. The amounts of radioactivity incorporated into Q43 and Q53 were not determined.

Time Course of Protein Changes. To investigate whether observed changes were transient or long lasting, the pattern of protein synthesis was examined 30 and 150 min after serum stimulation. Incorporation of [^{35}S]methionine into proteins N26, N28, and N47 (but not into protein N45) was already detectable at 30 min (Fig. 2*A*). In addition, large increases in many of the Q proteins (Q23, Q42, Q49, Q54, Q69, Q72, and Q98) along with decreases in Q145, Q176, and Q178 had taken place by this time. At 150 min, the pattern of protein synthesis looked very similar to that at 60 min, except for a few notable changes (Fig. 2*B*). Protein N26 was no longer detectable, and the amount of radioactivity incorporated into N47 was significantly reduced. N45 appeared to be modified during this same period so that it was possible to discern what might be two derivatives. In addition to these changes, the synthesis of a new protein, N56, and the sudden increase in the amount of [^{35}S]methionine incorporated into Q121 could be observed at 150 min. Thus, all the new proteins appear to have a unique pattern of synthesis; on the other hand, those Q proteins which began to increase at 30 min continued to increase throughout the first 150 min after serum stimulation.

Transcriptional and Translational Control. To resolve the question of whether the expression of these proteins was controlled at the transcriptional or translational level, cells were stimulated in the presence of actinomycin D, a potent inhibitor of transcription (9, 10). The increase in the amount of [^{35}S]methionine incorporated into new protein was unaffected by the presence of actinomycin D during the first 60 min after serum stimulation (Table 2). Thus, during the time that many of the changes in the pattern of protein synthesis were observed, the inhibition of transcription had no effect on overall rates of protein synthesis.

The effect of actinomycin D on the synthesis of specific proteins was examined by stimulating cultures for 60 min in the presence of the inhibitor and analyzing the proteins on two-dimensional polyacrylamide gels. The increased incorporation of [^{35}S]methionine into proteins N26, N45, N47, Q31, Q42, Q53, and Q72 appeared to be completely inhibited by the drug (Fig. 3). In contrast, the increased incorporation of [^{35}S]methionine into proteins N28, Q23, Q43, Q54, Q69, and Q98 was unaffected, and Q49 appeared to be induced to an even higher level. Decreases observed in proteins Q145, Q176, and Q178 also were unaffected by actinomycin D. Thus, the data indicate that approximately one half of the increases observed in serum-stimulated cells are due to alterations in the expression

Table 2. Rate of incorporation of [^{35}S]methionine into cytoplasmic protein

	[^{35}S]Methionine incorporation	
Cells	cpm/1×10^4 cells	%*
Quiescent	2.7×10^5	23
Quiescent + act. D	2.5×10^5	21
30 min + serum	4.8×10^5	40
30 min + serum + act. D	5.1×10^5	43
60 min + serum	6.0×10^5	50
60 min + serum + act. D	5.8×10^5	48
150 min + serum	12.0×10^5	100
150 min + serum + act. D	5.5×10^5	46

Cultures were pulse-labeled in conditioned media with [^{35}S]-methionine for 20 min at the times indicated, and the amount of protein present in each fraction was determined. Serum-stimulated 30-min, 60-min, and 150-min cultures were pulse-labeled from 20–40, 50–70, and 140–160 min, respectively. Actinomycin D (act. D) (2 μg/ml) was added to samples 15 min prior to the addition of serum.
* Percentage of incorporation into serum-stimulated 150-min cultures.

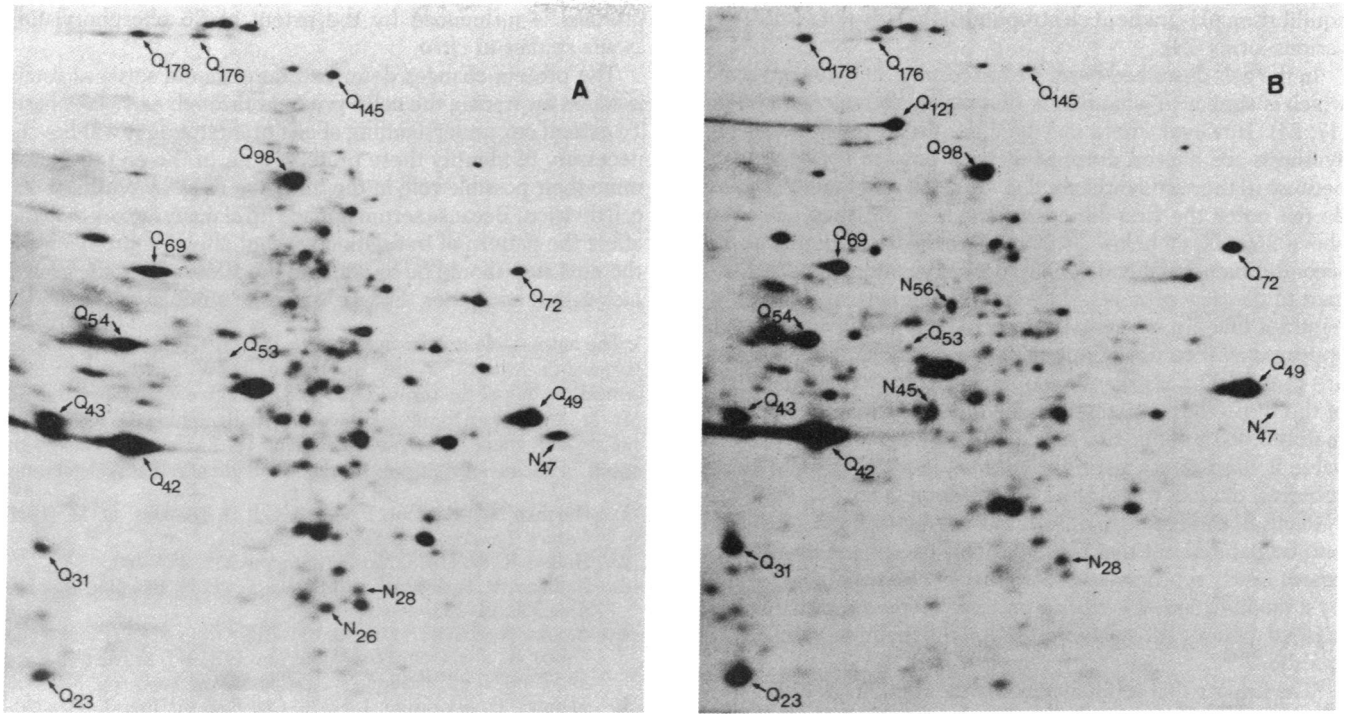

FIG. 2. Two-dimensional fluorograms of [^{35}S]methionine-labeled cytoplasmic proteins from 30-min (*A*) and 150-min (*B*) serum-stimulated cells. Parallel cultures were pulse-labeled with [^{35}S]methionine, and the labeled proteins were analyzed as described in Fig. 1. Serum-stimulated 30- and 150-min cultures were pulse-labeled in the intervals from 20–40 and 140–160 min, respectively.

of mRNA at the transcriptional level, and the remaining half are due to alterations of mRNA expression at the translational level. The results from Fig. 3 are summarized in Table 3.

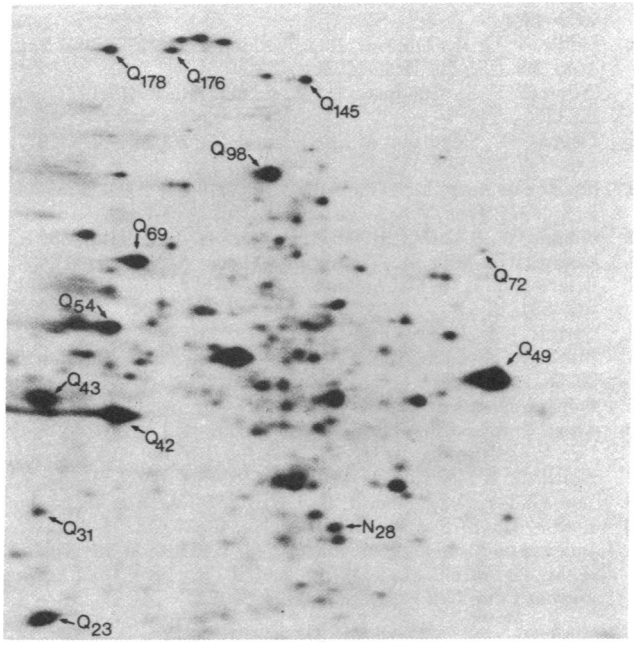

FIG. 3. The effect of actinomycin D on the pattern of protein synthesis. [^{35}S]Methionine pulse-labeled cytoplasmic proteins derived from a 60-min serum-stimulated culture were examined by two-dimensional gel electrophoresis as described in Fig. 1. The culture was pulse-labeled in the interval from 50–70 min after addition of serum. Actinomycin D (2 μg/ml) was added to the cells 15 min prior to the addition of serum.

DISCUSSION

Two reports have described changes in the synthesis of one or more proteins after serum stimulation of quiescent 3T3 cells (15, 16). Gates and Friedkin (15) have found that a M_r 50,000 protein(s) increases during lag phase, reaching a maximum by middle lag phase. It is likely that the changes we observe in proteins N45, N47, Q42, Q43, Q49, Q53, and Q54 correspond to the changes that they have reported in this area of their one-dimensional gel. Riddle *et al.* (16), using a similar method, have found changes in the amount of [^{3}H]isoleucine incorporated into several cytoplasmic proteins after serum stimulation; proteins in the range of M_r 33,000, 42,000, and 57,000 have increasing incorporation values, whereas proteins in the M_r 200,000 range have decreasing values. Again these findings are in agreement with the changes in specific proteins that we report here. Riddle *et al.* also have shown that one of the proteins migrating at M_r 42,000 on one-dimensional gels comigrates with actin on two-dimensional gels. An increase in actin (Q42) is also in agreement with our findings (Figs. 1 and 2; Table 1). We have not yet identified which species of actin is increasing because non-

Table 3. Effect of actinomycin D on specific cytoplasmic proteins

Q23	0	Q54	0
N26	+	N56	+ (NS)
N28	0	Q69	0
Q31	+	Q72	+
Q42	+	Q98	0
Q43	0	Q121	+ (NS)
N45	+	Q145	0
N47	+	Q176	0
Q49	0	Q178	0
Q53	+		

+, Actinomycin D sensitive; 0, actinomycin D insensitive; NS, data not shown.

equilibrium pH-gradient electrophoresis gels do not resolve the various forms (24).

In the gels described here, we can resolve 300–400 proteins, which is similar to what others find using the same gel system (17, 24). However, there may be other proteins whose rates of synthesis are altered during this time but are not detectable because (*i*) they are synthesized in too small an amount, (*ii*) they do not enter the first-dimensional gel, or (*iii*) they have M_rs above 250,000 or below 14,000 (approximate M_r limits of the second-dimensional NaDodSO$_4$/polyacrylamide gel). With regard to the new proteins, they are defined here as those polypeptides that are not visually detectable in quiescent cells but appeared after serum stimulation. It may be that these proteins are present but, as mentioned, are below the detection limit of the film. The lowest detectable proteins contained approximately 0.003% of the total radioactivity applied to the gel. If this value is used to estimate the minimal increase in the N class proteins, the following values are obtained: N26, 6.0; N28, 6.2; N45, 12.5; and N47, 15.1. (N56 was not determined.) It should also be pointed out that these proteins do not necessarily represent newly synthesized proteins; they also may be generated by a modification of a second protein. *In vitro* translation, described below, of isolated mRNAs will help to resolve this question.

The finding that actinomycin D either inhibits or completely blocks the increased synthesis of proteins N26, N45, N47, N56 (data not shown), Q31, Q42, Q53, Q72, and Q121 (data not shown) argues that the expression of these proteins requires the synthesis of new mRNA. However, another possibility is that these mRNAs are already present as part of the preexisting nonpolysomal pool of mRNA and that their activation requires instead synthesis of a new protein(s). It is then the expression of this protein(s) that is under transcriptional control. In order to discriminate between these two possibilities, one should analyze nonpolysomal and polysomal mRNA from quiescent and stimulated cells incubated in the absence and presence of actinomycin D in cell-free lysates (25–28). By using this approach, one also should be able to determine the fate of the mRNAs coding for proteins Q145, Q176, and Q178.

The increased synthesis of proteins N28, Q23, Q43, Q49, Q54, Q69, and Q98 in the presence of actinomycin D suggests that these proteins are coded for by the pool of preexisting nonpolysomal mRNA (9–11). The mechanism by which their expression is controlled is unknown. It has been reported by Lee and Englehardt (29) that in Vero cells (monkey fibroblasts) grown to stationary phase, there is a large pool of nonpolysomal mRNA that is quantitatively different from the mRNA that is being translated. The two classes of mRNA do not differ with respect to either 3′-polyadenylylation or the 5′-cap structure. However, these findings, do not exclude differences in individual mRNA molecules or cell type variability. Another possibility is that serum stimulation leads to an alteration in some other component of the translational apparatus that changes the affinity of ribosomes for mRNA (28, 30). The phosphorylation of 40S ribosomal protein S6 could be such an event. It is clear from our earlier findings that the phosphorylation of S6 temporally correlates with the expression of the translational control proteins listed above (7, 8). Whether the expression of these proteins is influenced by the extent of S6 phosphorylation awaits studies *in vitro*.

The protein changes described here should serve as useful markers for tracing the cells' progress through early lag phase. To extend our understanding of cell proliferation it will now be necessary to identify these proteins and, in the end, to determine their possible role in the initiation of DNA synthesis and cell division. Because serum is made up of many factors that may affect the pattern of translation without affecting proliferation, the next step should be to examine the affect of specific growth factors and hormones that are known to stimulate growth (31).

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