Cell-Cycle-Specific and Serum-Dependent Expression of Gamma-Actin mRNA in Swiss Mouse 3T3 Cells

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Received 2 November 1987/Accepted 2 March 1988

We isolated cDNA clones that represent genes whose expression is enhanced when resting Swiss mouse 3T3 cells are stimulated to proliferate with serum. Two clones (designated pME₁ and pMR₆) were analyzed further. A partial sequence analysis of the pME₁ insert DNA indicated that it contained a 104-base-pair stretch with extensive homology to the 3' untranslated region of gamma actin. Similar analysis of the insert DNA from the pMR₆ clone indicated that it did not correspond to any previously reported gene sequence. We used the pME₁ clone as a probe to determine the level of gamma actin-specific transcript in 3T3 cells under a variety of conditions. The level of gamma actin-specific mRNA began to increase in resting cells upon serum stimulation and reached a peak at 6 h. Thereafter its level declined, and by 24 h it was hardly detectable. In contrast, pMR₆-specific transcript was detectable in resting cells but remained elevated even at 24 h poststimulation. The level of gamma-actin mRNA was elevated in resting cells by 12-*O*-tetradecanoylphorbol-13-acetate, calcium ionophore A23187, and bombesin and to a lesser extent by cholera toxin, fibroblast-derived growth factor, and dibutyryl cyclic AMP. However, insulin, vasopressin, or epidermal growth factor failed to enhance gamma-actin mRNA levels in resting cells. Inhibitors of transcription diminished the induction of gamma-actin mRNA. Gamma-actin gene was superinduced in serum-stimulated cells by cycloheximide, an inhibitor of translation. Analysis of proteins from serum-stimulated cells by two-dimensional gel electrophoresis indicated that enhanced transcription of gamma-actin mRNA resulted in a concomitant increase in the corresponding actin protein. The possible role of gamma actin, a component of the cytoskeleton, in the regulation of cell growth is discussed.

A number of events are initiated before the onset of DNA synthesis in resting Swiss mouse 3T3 cells when they are stimulated to proliferate by using serum (for reviews see references 2 and 30). Molecular cloning of cDNAs corresponding to mRNAs expressed during the cells' transit through G₁ has been the recent approach used to characterize the genes mediating the prereplicative events. Several groups have reported on the identification of such cell cycle-dependent genes. Cochran et al. (7) reported on five platelet-derived growth factor-inducible genes in BALB/c mouse 3T3 cells. Nathan's group reported on the expression of several genes which are activated soon after resting BALB/c 3T3 cells are stimulated with serum or platelet-derived growth factor (16, 17, 19–21). One of these genes, called proliferin, belongs to the prolactin growth hormone family (19). Hirschhorn et al. (15) identified five distinct cDNA clones by using a temperature-sensitive baby hamster kidney cell line which corresponded to mRNAs which are enhanced when resting cells are stimulated to proliferate by using serum. One of these genes was identified later to be the vimentin gene (11). Edwards et al. (9), using approaches similar to those used by others, have characterized five G₁-specific genes in Swiss mouse 3T3 cells. Subsequently, one of these genes was reported to share sequence homology with a gene for human collagenase inhibitor (10).

We have been interested in identifying genes that regulate cellular proliferation. To this purpose we have constructed a cDNA library derived from poly(A)⁺ mRNAs isolated from Swiss mouse 3T3 cells that were stimulated to proliferate by

using serum. We screened recombinants in the library to identify those clones whose corresponding transcripts are enhanced in serum-stimulated cells. The present communication describes the properties of two such clones. One of them, pME₁, contains the highly conserved 3' untranslated (UT) region of gamma actin and was expressed in early G₁ phase of the cell cycle. Using pME₁ as a probe, we have determined some features regulating the enhanced expression of gamma actin in resting cells stimulated to proliferate with serum. The possible role of gamma actin in cellular proliferation is discussed.

MATERIALS AND METHODS

Cell cultures. All tissue culture supplies were from GIBCO Laboratories. Stock cultures of Swiss mouse 3T3 cells were propagated using Dulbecco modified Eagle medium (DMEM) supplemented with 10% calf serum (37). Cultures were rendered quiescent by incubating cells (seeded at 3 × 10⁵ cells per 10-cm dish) in DMEM containing 10% calf serum for 5 days, followed by incubation for 2 days in DMEM with 0.5% calf serum. The resting cells were stimulated using medium containing 10% calf serum as described previously (37).

Enzymes and chemicals. All restriction enzymes were purchased from Bethesda Research Laboratories, International Biotechnologies Inc., or Boehringer Mannheim Biochemicals and were used according to the manufacturers' directions. Avian myeloblastosis virus reverse transcriptase, *Escherichia coli* DNA polymerase I, ionophore A23187, actinomycin D, and 5,6-dichloro-β-D-ribofuranosyl-benzimidazole (DRB) were purchased from Boehringer Mann-

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heim Biochemicals or from Calbiochem-Behring. [32 P]dCTP (3,000 Ci/mmol) was purchased from ICN Radiochemicals. The following were purchased from Sigma Chemical Corp.: cycloheximide, hydroxyurea, sodium butyrate, insulin, vasopressin, bombesin, dibutyl cyclic AMP (cAMP), cholera toxin, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The following were obtained from Collaborative Research: epidermal growth factor (EGF), fibroblast growth factor (FGF), and oligo(dT)-cellulose. Fibroblast-derived growth factor was a gift from Enrique Rozengurt.

RNA isolation. Guanidine isothiocyanate lysate of whole cells was used for the isolation of RNA by the procedure of Chirgwin et al. (5). Poly(A) $^{+}$ RNA was selected by two cycles of binding to oligo(dT)-cellulose (1).

Construction of cDNA library from serum-stimulated cells and screening for cDNA clones. Quiescent Swiss mouse 3T3 cells were stimulated to proliferate by using serum. The cultures were harvested 8 h after stimulation, and total cellular RNA was isolated. The poly(A) $^{+}$ RNA fraction from the total cellular RNA was used as a template for the synthesis of double-stranded cDNAs, which were then inserted into the Okayama-Berg vector (27, 28). The resultant recombinant molecules were used to transform competent *E. coli* (strain JA221) cells, generating a cDNA library of approximately 5×10^4 recombinants.

Colony hybridization. The colony hybridization procedure was identical to that described by Linzer and Nathans (20). Labeled [32 P]cDNA probes were synthesized with a specific activity of 10^8 cpm/ μ g of cytoplasmic poly(A) $^{+}$ RNA.

RNA blot analysis. Total cytoplasmic RNA was denatured using 6.3% formaldehyde–50% formamide and then fractionated on a 1% agarose gel containing 6.6% formaldehyde (23). Blotting of the nucleic acid to Gene Screen (New England Nuclear Corp.) and hybridization with 32 P-labeled nick-translated probes were done as described by Thomas (38) or by the manufacturer. The recombinant plasmids and the corresponding DNAs were isolated and purified by methods described by Maniatis et al. (23). Purified DNAs were nick translated to 1×10^8 to 2×10^8 cpm/ μ g by methods described by Rigby et al. (33). The blots were prepared for rehybridization according to the procedure recommended by the manufacturer.

Sequence analysis of the pME $_1$ cDNA clone. DNA was sequenced by using the Sanger dideoxy method (36). The cDNAs were subcloned into M13mp19 (25) and then sequenced. The sequencing gels were 8% polyacrylamide containing 7 M urea in Tris-borate buffer. The September 1987 updated version of the National Institutes of Health GenBank data base was searched for sequence homologies, using the Micro Genie sequence analysis program.

[35 S]methionine labeling and two-dimensional polyacrylamide gel electrophoresis. Swiss mouse 3T3 cells were seeded in 96-well tissue culture plates (Nunc) at a concentration of 10^4 per well in DMEM and 10% calf serum. The cultures were confluent 2 days later, at which time the medium was changed to DMEM with 0.5% calf serum. After 48 h the cells were labeled in 0.1 ml of DMEM (methionine free) with or without 10% dialyzed calf serum and containing 1 μ Ci of [35 S]methionine (1,110 Ci/mmol). Some of the cultures stimulated with 10% serum received actinomycin D at a concentration of 5 μ g/ml. The cultures were labeled for 8 h, after which time they were washed three times with cold saline and immediately dissolved in 25 μ l of urea-lysis buffer containing Nonidet P-40 and quick-frozen. Cell extracts were obtained, and samples containing 7.5×10^5 acid-precipitable cpm were analyzed using two-dimensional poly-

FIG. 1. Sequence homology between pME $_1$ and the reported 3' UT region of mouse (18), bovine (8), and human (6) gamma-actin sequences. Solid line represents vector sequences, and restriction sites are indicated as follows: B, *Bam*HI; P, *Pst*I; H, *Hind*III. Dots indicate nucleotide identity; dashes represent gaps that have been introduced to align best the sequence data. The actual nucleotide is indicated (G, C, A, T) where there is a difference in the nucleotide sequence.

acrylamide gel electrophoresis as described by O'Farrell (26). The isoelectric focusing gel contained 1.6% pH 5–7 and 0.4% pH 3.5–10.0 ampholytes (LKB). The final pH gradient was between pH 7.10 and pH 4.4. The second-dimension gel was an 8.5 to 12.5% linear gradient. The protein standards used were from Bio-Rad.

RESULTS

Differential expression of mRNAs in resting and serum-stimulated cells. The cDNA library was surveyed for clones containing cDNAs whose corresponding genes were differentially expressed when resting Swiss 3T3 cells were stimulated to proliferate by using serum. Five thousand clones were screened for differential hybridization, using 32 P-labeled single-stranded cDNA synthesized with mRNAs from resting or stimulated cells. Two clones, pME $_1$ and pMR $_6$, contained DNA sequences whose corresponding mRNAs were differentially expressed in resting cells. Partial sequence analysis indicated that the pME $_1$ insert DNA contained a 104-base-pair stretch which had extensive homology with the 3' UT region of gamma-actin mRNA. The partial sequence of the pME $_1$ insert from the 3' end was compared with the corresponding regions of mouse (18), bovine (8), and human (6) gamma actin (Fig. 1). Ponte et al. (31) reported that this region of the actin gene is isotype specific and is highly conserved between species. Therefore, the cDNA clone pME $_1$ contains an insert corresponding to the gamma-actin gene. The cDNA clone pMR $_6$ contained a 600-base-pair insert. DNA sequence analysis of this insert (data not shown) indicated that its sequence is not related to any of the genes in the National Institutes of Health sequence library (September, 1987).

Serum stimulation of gamma-actin mRNA. The cDNA clone pME $_1$ contained the 3' UT region isotype specific for gamma actin. This permitted us to use this plasmid as a probe to analyze the level of gamma-actin mRNAs in cells. In subsequent discussions we will refer to the pME $_1$ cDNA as the gene for mouse gamma actin. Figure 2 shows a Northern (RNA) blot analysis of total cytoplasmic RNAs isolated from resting (quiescent) 3T3 cells and resting 3T3 cells incubated with medium containing 10% serum (stimulated). The same blot was used sequentially for hybridization with the two probes pME $_1$ and pMR $_6$, and they hybridized to transcripts of 2.6 and 1.7 kilobases (kb), respectively. Densitometric measurements indicated that the relative increase in the level of transcript in serum-stimulated as compared with resting cells was 18-fold for gamma actin and 2-fold for pMR $_6$.

FIG. 2. Identification of transcripts corresponding to cDNA clones pME₁ (gamma actin) (A) and pMR₆ (B). Total cytoplasmic RNA was isolated at 8 h after stimulation of quiescent cells with (stimulated) or without (quiescent) 10% calf serum. Equal amounts (20 μ g per lane) of RNA were subjected to electrophoresis on agarose gel and transferred to Gene Screen as described in Materials and Methods. The blot was hybridized with the nick-translated pME₁ probe (specific activity, 10^8 cpm/ μ g of DNA). The same blot was used for rehybridization with nick-translated pMR₆ DNA (10^8 cpm/ μ g). The blots were exposed to X-ray film for 24 h. Size standards used were 28S and 18S rRNAs.

The ability of resting cells to initiate DNA synthesis is dependent on the concentration of serum in the growth medium (3). Maximal induction of DNA synthesis occurs in resting Swiss mouse 3T3 cells using growth medium containing 8 to 10% serum. Therefore, it was of interest to determine whether the increased expression of gamma-actin gene is also dependent on the concentration of serum in the growth medium. Resting cells were stimulated to proliferate by using medium containing various concentrations of serum. Cultures were harvested at h 6, and RNA samples were obtained and used for Northern blot analysis. The hybridization signals obtained using nick-translated gamma actin, pMR₆, and pMC₃ cDNA clones are shown in Fig. 3. Clone pMC₃ was used as a control in this and subsequent experiments since it gave no differential hybridization in the colony or dot-blot analyses. The autoradiograms were analyzed by densitometry. In this experiment there was a detectable level of gamma actin-specific mRNA in cells receiving no serum. Compared with such cells, the extent of increase in gamma-actin mRNA levels was 3-, 8-, 12-, and 16-fold for 0.1, 1.0, 5, and 10% serum concentrations, respectively (Fig. 3A). Thus the rise in gamma-actin RNA level in stimulated cells varied with the concentration of serum in the growth medium. The pMR₆ RNA level was enhanced twofold in stimulated cells as compared with resting cells, and the observed rise was maximal in cells receiving 1% serum (Fig. 3B). The autoradiogram shown in Fig. 3B contained an RNA species of 2.4 kb in addition to the predominant species of 1.7 kb. The intensity of this band did not change significantly with the concentration of serum in the growth medium. The presence of this 2.4-kb transcript in total cellular RNA was not observed consistently. The control clone, pMC₃, hybridized to a 5.0-kb RNA. The relative level of this transcript did not change significantly with different concentrations of serum.

Cell cycle-specific expression of gamma actin and pMR₆. Swiss mouse 3T3 cells enter the G₀/G₁ phase when they are deprived of serum or exogenous growth factors. Several investigators have shown previously that addition of serum

FIG. 3. Dependence of mRNA levels in Swiss 3T3 cells on the concentration of serum in the growth medium. Total cytoplasmic RNA was isolated from quiescent cells at 8 h after stimulation with various concentrations of calf serum (0.0, 0.1, 1.0, 5.0, and 10.0%). Equal amounts (20 μ g) of total cytoplasmic RNA were separated on a 1% agarose gel, and the blot was hybridized sequentially using the following probes: (A) pME₁ (gamma actin); (B) pMR₆; (C) pMC₃. The specific activity of all three DNA probes used was adjusted to 10^8 cpm/ μ g. Each blot was exposed to X-ray film for 24 h.

or growth factors to such resting cells triggers an increase in the rate of initiation of DNA synthesis after a lag phase of 10 to 12 h (for reviews, see references 30 and 35). On the basis of results obtained from experiments involving [³H]thymidine incorporation and autoradiography (results not presented), we have determined in this study that the resting cells enter S phase after a 10-h lag period subsequent to serum stimulation. This period represents the G₁ phase of the cell cycle. The cDNA clones were used as probes to monitor the levels of the corresponding RNA species in quiescent cells at various times after serum stimulation. The results of a Northern blot analysis from such an experiment

FIG. 4. Time course of appearance of specific transcripts in resting cells after stimulation with serum. Quiescent 3T3 cells were stimulated to proliferate by using 10% calf serum. At time 0, some of the serum-stimulated cells also received 10 μ g of cycloheximide per ml. RNA was isolated from the cultures at 0, 3, 6, 8, 12, and 24 h after stimulation. Equal amounts (20 μ g) of RNA were electrophoresed, and the blot was sequentially probed with (A) pME₁ (gamma actin), (B) pMR₆, or (C) pMC₃. The rest of the details were identical to those described in the legend of Fig. 3. Lanes labeled 6', 8', or 12' indicate the RNA sample obtained at 6, 8, or 12 h, respectively, from cultures incubated with cycloheximide.

FIG. 5. Effect of inhibitors upon the induction of pME₁ and pMR₆. Resting cells were stimulated with 10% calf serum. The various inhibitors were added to the cultures at this time, and the cytoplasmic RNA was obtained from cells incubated for 6 h after stimulation. RNA blot analysis was carried out as described for previous figures. A 20- μ g sample of total RNA was used per lane. Q, Quiescent cells; S, 10% calf serum. Inhibitors: HU, 2 mM hydroxyurea; NaB, 5 mM sodium butyrate; Cyc, 10 μ g of cycloheximide per ml; Act D, 5 μ g of actinomycin D per ml; DRB, 25 μ g/ml. The blot was sequentially hybridized with nick-translated pME₁ (gamma actin) (A) and pMR₆ (B) probes.

are shown in Fig. 4. Densitometric analysis indicated that, compared with resting cells, a 15-fold increase in gamma-actin transcript occurred at 6 h after serum stimulation; thereafter, it began to decrease and was undetectable by 24 h. Addition of cycloheximide to resting cells at the time of serum stimulation enhanced the level of gamma-actin transcript (compare lanes 6 and 6', 8 and 8', and 12 and 12' in Fig. 4A). In contrast, the rise in pMR₆ RNA was only twofold over that in resting cells, and the maximal increase in cells occurred at 8 h after serum stimulation. Cycloheximide inhibited the observed increase in the level of pMR₆ RNA (lanes 6 and 6', 8 and 8', and 12 and 12' in Fig. 4B). Additionally, the pMR₆ RNA level remained high even at the 24-h time point. Figure 4C represents a control hybridization using cDNA clone pMC₃; the corresponding RNA levels remained relatively unchanged at the various time points.

Effects of inhibitors on the activation of gamma actin and pMR₆ expression. Changes in mRNA levels can be effected by enhanced transcription or posttranscriptional changes, affecting mRNA stability. Inhibitors of transcription or translation can be used to determine the level of control. Cycloheximide, an inhibitor of protein synthesis, had a differential effect on the levels of gamma actin- and pMR₆-specific transcripts (Fig. 4). To determine whether gamma actin or pMR₆ is regulated transcriptionally, we determined the effect of two well-known inhibitors of transcription, actinomycin D and DRB. Additionally, we tested the effect of inhibitors of DNA synthesis (hydroxyurea and sodium butyrate). All the inhibitors were added to the quiescent cultures at the time of serum stimulation. The cultures were harvested 6 h later, and the RNA samples obtained were analyzed by Northern blot. Figure 5 presents such an analysis using the same probes as described in the previous section. The results show that gamma actin- and pMR₆-specific RNA levels were affected differentially by the various inhibitors. Compared with resting cells, the gamma actin-specific RNA level increased in stimulated cells. This increase was inhibited by actinomycin D or DRB. After correcting for the signal generated by RNA from resting cells, we estimated the degree of inhibition by actinomycin D and DRB to be 90 and 98%, respectively. The observed twofold increase in pMR₆ RNA level in serum-stimulated as compared with resting cells was inhibited by both drugs. However, as seen in the previous section and in the present

FIG. 6. Induction of gamma actin by various agents. (A) Resting cells were incubated with growth medium containing the following: Q, 0.5% calf serum; S, 10% calf serum; E, 10 ng of EGF per ml; I, 1.0 μ g of insulin per ml; V, 10 ng of vasopressin per ml; EIV, EGF + insulin + vasopressin; F, 50 ng of FGF per ml; FI, FGF + insulin; TPA, 50 ng/ml; A23187, 5 μ M of the calcium ionophore. (B) Q, 0.5% calf serum; S, 10% calf serum; CT 100 ng of cholera toxin per ml; I and TPA, same as for panel A; BB, 6 nM bombesin; FDGF, 0.25 μ g of fibroblast-derived growth factor per ml; cAMP, 2 mM; CT + I, cholera toxin + insulin; TPA + I, TPA + insulin. The two panels represent two different experiments using two different batches of resting cells. In all instances, cells were harvested 6 h after stimulation, and cytoplasmic RNA was obtained. Northern blot analysis was carried out using equal amounts of RNA (20 μ g) per lane in the gel. The blots were probed with nick-translated pME₁ cDNA (gamma actin). The rest of the details are identical to those described for Fig. 2.

experiment, cycloheximide had a differential effect on gamma-actin and pMR₆ RNA levels: the former was enhanced, while the latter was partially suppressed. Expression of either gene was unaffected by hydroxyurea or sodium butyrate, a finding consistent with the notion that enhanced expression of both genes is a prereplicative rather than a postreplicative event.

Effect of growth factors, tumor promoters, and ionophore. Several growth factors or well-defined agents, when used in appropriate combinations, can induce DNA synthesis in resting Swiss mouse 3T3 cells (for a review, see reference 35). These include polypeptide hormones like EGF, FGF, fibroblast-derived growth factor, insulin, and vasopressin; well-defined agents like cholera toxin, the amphibian tetradecapeptide bombesin, and cAMP; and tumor promoters such as TPA or the calcium ionophore A23187. When used singly, these agents, except bombesin, do not induce DNA synthesis in resting Swiss 3T3 cells (35). However, in combination they exhibit synergistic interactions culminating in DNA synthesis in cell cultures maintained in medium containing no serum (35). Therefore, we examined whether any of these factors could enhance the level of gamma actin in resting 3T3 cells. Experiments involving the incorporation of [³H]thymidine into cells indicated that none of the agents tested, excluding bombesin, could stimulate DNA synthesis in cells when used singly (results not presented). However, when used in combination, some of the agents induced synthesis in cells. Such combinations included EGF plus insulin and vasopressin, FGF plus insulin, cholera toxin plus insulin, cAMP plus insulin, or TPA plus insulin. These results are similar to those reported previously by others (for a review, see reference 35). Of the agents used, TPA, calcium ionophore A23187 (Fig. 6A), and bombesin (Fig. 6B) enhanced the level of gamma actin-specific transcript as efficiently as did serum in resting cells. The level of gamma-actin RNA was enhanced by cholera toxin, fibroblast-de-

FIG. 7. Gamma- and beta-actin mRNA levels in resting cells after serum stimulation. Quiescent cells (Q) were stimulated with medium containing 10% calf serum, and total cytoplasmic RNA was isolated 6, 12, and 24 h post-serum stimulation. Equal amounts (20 μ g) of RNA were subjected to electrophoresis, and the blot was sequentially probed with (A) gamma actin (pME₁) and (B) beta actin (pHF β A-3'UT).

rived growth factor, or cAMP to a lesser extent than by serum stimulation (compare lanes marked CT, FdGF, and cAMP in Fig. 6B). Interestingly, insulin was found to suppress the activation of gamma-actin RNA in resting cells (compare lanes marked CT and CT+I or TPA and TPA+I). It is also significant that there was no increase in the level of gamma actin in resting cells incubated with a combination of certain growth factors which induces DNA synthesis (compare lanes marked FI and EIV in Fig. 6A).

Transcriptional activation of gamma- and beta-actin genes in 3T3 cells. Edwards et al. (9) reported that there is an increase in the concentration of beta actin when resting cells traverse the G₁ period. Therefore it was of interest to compare the increase in the levels of both gamma- and beta-actin transcripts in the same cell population during its transit through G₁. Figure 7 is a Northern blot of RNAs obtained at various times after quiescent cells were stimulated with serum. As observed in previous experiments, the level of gamma-actin mRNA increased in resting cells at 6 h (17-fold) after serum stimulation and decreased thereafter (Fig. 7A). The same RNA blot, when hybridized with the beta-actin probe, showed the presence of detectable levels of corresponding transcripts in resting cells (Fig. 7B). In addition to the 1.9-kb beta-actin transcript, the quiescent cells contained an additional RNA species of 3.8 kb in size. At present the significance of the 3.8-kb transcript is not clear. The level of beta-actin mRNA increased by 12 h after serum stimulation and remained high through the 24-h time period. Compared with the marked elevation and subsequent disappearance of gamma-actin mRNA in the cells after serum stimulation, the level of beta-actin transcript increased only by 75% of that found in resting cells.

Enhanced synthesis of gamma actin in resting cells after serum stimulation. The results presented in the previous section suggest that in resting cells stimulated with serum, gamma-actin mRNA accumulates to a greater level than that of beta-actin mRNA. Therefore it was of interest to determine whether the increase in mRNA level resulted in a concomitant rise in the level of gamma-actin protein. To ascertain this possibility, resting cells were labeled with [³⁵S]methionine for 8 h in the absence or presence of 10% serum. Some cultures receiving serum received actinomycin D also at the time of stimulation to inhibit transcription. The various actin isotypes were separated by two-dimensional gel electrophoresis on polyacrylamide gels. The two isotypes of actin, gamma and beta, could be resolved on the basis of the direction of isoelectric focusing as reported by Gerstenfeld et al. (14). Compared with beta actin, the level of gamma-actin protein was low in quiescent cells (Fig. 8A). However, after serum stimulation the level of gamma actin increased (Fig. 8B). Such a rise in the level of gamma-actin protein was suppressed when serum-stimulated cells were

stretch of sequence homologous to the 3' UT region of mouse (18), bovine (8), or human (6) gamma-actin gene. Ponte et al. (31) reported that this region is isotype specific for the various actins and that it is highly conserved between species. Therefore the gene corresponding to the cDNA clone pME₁ is that encoding mouse gamma actin. DNA sequence analysis of the insert from the pMR₆ cDNA clone indicated that it did not correspond to any known genes.

Since the pME₁ clone contained only the 3' UT region specific for gamma actin, we were able to use this plasmid as a probe for examining its expression during the cell cycle and its ability to be stimulated by various mitogens. Gamma-actin mRNA synthesis reached a peak level at 6 h after serum stimulation and then declined rapidly (Fig. 4). The addition of cycloheximide to the serum-stimulated cultures enhanced the transcript level in the cell. Cycloheximide inhibited protein synthesis in resting cells in the presence or absence of serum by 90 to 95% over the control. Transcriptional inhibitors like actinomycin D or DRB inhibited the serum-dependent increase in the level of gamma-actin transcript. The observed increase in the level of transcript was reflected also in the enhanced synthesis of the gamma-actin protein. In contrast, neither the level of beta-actin mRNA nor that of the corresponding protein increased to the same degree as that of gamma actin (Fig. 7 and 8). The present results concerning the response of the gamma-actin gene to various inhibitors do not permit us to determine whether there is an actual increase in the rate transcription of the gene in resting cells after serum stimulation. Apparently, a protein, newly synthesized or with a short half-life, represses gamma-actin gene transcription or decreases the stability of the mRNA in resting cells. Serum growth factors may overcome such a repression, a conclusion consistent with the action of serum and cycloheximide in resting cells. Rittling et al. (34) described nine cell cycle genes whose induction in resting cells was dependent upon serum but independent of proteins synthesis. According to these authors, the activation of these genes represents a cell's primary response to serum and growth factors. In light of the present results, the gamma-actin gene should be included in the above group of cell cycle-dependent genes. Gamma actin may represent an early gene in the genetic program for cell growth. In contrast, the other cDNA clone (pMR₆) may correspond to a gene whose properties fit to those of a late G₁ gene, since the observed increase in its transcript level was inhibited by cycloheximide, actinomycin D, or DRB.

It is now becoming increasingly clear that the cellular cytoskeleton may play a role in initiation of DNA synthesis and cell division. Colchicine and colcemid, drugs capable of depolymerizing microtubules, markedly enhance the stimulatory effects of many growth factors on DNA synthesis in Swiss mouse 3T3 cells (12, 24, 29), an observation which is consistent with the above notion. Maness and Walsh (22) reported that dihydrocytochalasin B blocks initiation of DNA synthesis in resting Swiss mouse 3T3 cells stimulated with serum, with EGF and insulin, or with a combination of other mitogens. These results suggest a possible involvement of actin in the initiation of DNA synthesis.

Several examples exist in the literature illustrating changes in gamma- and beta-actin levels in cells. Gates and Friedkin (13) reported on the appearance of a protein with *M_r* of 50,000 when resting cells were stimulated to proliferate. A similar observation was reported by Riddle et al. (32), who suggested that the level of the actin protein is increased during the cells' traverse from G₀/G₁ to S. In the present study we have shown that, unlike beta actin, the level of

gamma actin-specific transcript increased transiently during the G₁ period and decreased thereafter. Such an increase in mRNA level was accompanied also by an increase in the corresponding protein. The level of gamma actin increases fourfold in bovine lymphocytes stimulated with phytohemagglutinin (8). The ratio of the levels of beta- to gamma-actin protein decreases in the rat pheochromocytoma PC12 cell line induced to differentiate by nerve growth factor (4). Similarly, the transcript level of beta- and gamma-actin mRNAs changes during spermatogenesis in mouse testes (39). The transcript specific for gamma actin is abundant in early postmeiotic cell types and decreases in later postmeiotic cell types. The reverse is true for the level of beta actin. Thus both actin isotypes appear to be regulated differentially in cells during cellular proliferation and differentiation. In the present study we have shown that during cell cycle progression the two nonmuscle actins are regulated differentially.

The induction and repression of gamma actin in cells are regulated by several factors. Recently Ferrari et al. (11) reported on the increased expression of the vimentin gene, another cytoskeletal protein, in resting cells treated with TPA or serum. Mitogens like insulin, EGF, or platelet-poor plasma do not induce vimentin mRNA; these results are similar to those reported here concerning gamma-actin gene expression. Mitogens like serum and bombesin may generate a factor(s) in resting cells which could induce cytoskeletal proteins as well as initiate DNA synthesis. The activation of gamma actin as shown in the present study, as well as in the recent report concerning vimentin, supports this notion. Compounds like TPA, calcium ionophore A23187, and cholera toxin may generate factors which, although capable of inducing gamma-actin expression, fail to initiate DNA synthesis. Growth factors like EGF, insulin, FGF, or vasopressin, alone or in combination, do not induce gamma-actin gene expression despite the fact that such combinations can initiate DNA synthesis in resting cells. Indeed, when insulin was used in combination with TPA or cholera toxin, gamma-actin expression was inhibited even though these combinations induce DNA synthesis (Fig. 6A and reference 35). These results suggest that factors induced by TPA or cholera toxin in stimulating gamma-actin and some other cytoskeletal genes might be suppressed by insulin-induced factors. On the other hand, serum, a complex mixture of growth factors, can initiate gamma-actin expression and DNA synthesis. This could be the result of a variety of factors counteracting the inhibition of gamma-actin expression by insulinlike growth factors in serum-stimulated cells.

ACKNOWLEDGMENTS

We thank L. Kedes for the gift of plasmid pHFβA-3'UT. We also thank Ronald Cihlar for critically reviewing the manuscript and Adriana Cabrales for her skillful preparation of the manuscript.

These studies were aided by Public Health Service grant A120296 from the National Institutes of Health.

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