Control of Proliferin Gene Expression in Serum-Stimulated Mouse Cells

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The serum-inducible expression of proliferin genes in BALB/c 3T3 cells was found to be dependent on both protein synthesis and an extended presence of serum in the medium. Even though no mature proliferin mRNA was detected in serum-starved cells, transcription of the proliferin genes occurred in these resting-cell cultures, indicating that posttranscriptional events may be important for regulating proliferin mRNA levels. These results suggest that protein synthesis after serum stimulation of quiescent mouse fibroblasts is required for posttranscriptional processing or stabilization of proliferin RNA. Proliferin RNA levels were found to be heterogeneous among serum-stimulated cells analyzed by *in situ* hybridization. This heterogeneity is probably due to asynchrony in the population and may point to a correlation between the time of proliferin expression and the time of entry of a cell into S phase.

Serum stimulation of quiescent mouse fibroblasts results in elevated levels of several mRNAs. Some of these, including *c-fos*, *c-myc*, and other still unidentified mRNAs (3, 4, 7, 9, 11), appear rapidly and transiently in response to serum; another set of mRNAs, including proliferin (14) and p53 (21), increase in amount later during the G₁ period. Elevated levels of the rapidly induced mRNAs are due, at least in part, to increased transcription of these genes (7). Furthermore, these early events occur in the absence of protein synthesis, suggesting that they are primary responses to serum or growth factor stimulation of resting cells (4, 9, 11). The expression of these specific mRNAs in response to a growth stimulus suggests that their gene products are involved in the process, and perhaps the regulation, of cell growth. At least three of these genes (*fos*, *myc*, and p53) have oncogenic activity, lending further support to the theory of their involvement in regulating cell growth.

In contrast to these early mRNAs, proliferin mRNA levels are detectable at 3 h after serum stimulation and increase during mid- to late G₁, peaking at the onset of DNA synthesis, approximately 12 h after serum stimulation of resting BALB/c 3T3 cells (14). BALB/c 3T3 cells synthesize and secrete proliferin protein with a similar time course (11a); this protein appears to be identical to the mitogen-regulated protein secreted by Swiss 3T3 cells (19, 20). Either serum or purified platelet-derived growth factor added to quiescent BALB/c 3T3 cells is able to stimulate proliferin mRNA accumulation (14). The identification of proliferin as a member of the prolactin-growth hormone family (15) further supports a role for it in the growth process, since hormones in this family can stimulate cell growth (24). Proliferin, along with other members of this hormone family, including proliferin-related protein (17), placental lactogen I (23), and placental lactogen II (8a), is expressed specifically in mouse placental tissue (13), while prolactin and growth hormone synthesis occur in the anterior pituitary. Expression of proliferin in placental tissue *in vivo* and in embryo-derived cell lines in culture (e.g., BALB/c 3T3 [14], Swiss 3T3 [19], and C3H 10T1/2 [16]) indicates that proliferin may function in embryonic growth and development.

Except for some pituitary tumor-derived cell lines that continue to synthesize prolactin or growth hormone (25), proliferin is the only member of this family that has thus far been found to be expressed in permanent lines of cultured mouse cells. The cell lines that synthesize proliferin mRNA do not appear to be derived from cells expressing proliferin in the mouse, since proliferin mRNA is not detected in embryonic tissue (13) or in primary mouse embryo fibroblasts (20; Wilder and Linzer, unpublished observations). The mouse genome contains several copies of the proliferin gene, at least two of which are transcriptionally active in both placental tissue and cultured mouse cell lines, suggesting that similar mechanisms for regulating proliferin gene activity are operating both *in vivo* and in cell culture (27). In this study, we have analyzed the control of expression of the proliferin gene family in cultured BALB/c 3T3 cells.

MATERIALS AND METHODS

Cell culture. BALB/c 3T3 cells were grown in minimal essential medium supplemented with glutamine (2 mM), penicillin and streptomycin (10 U of each per ml), and 10% fetal bovine serum. Quiescent cultures of BALB/c 3T3 cells were generated by growing the cells to confluence and then maintaining them for 48 to 72 h in minimal essential medium containing 0.5% fetal bovine serum. Resting cells were stimulated by the addition of serum to 20%. BALB/c 3T3 cell cultures were treated with 10 µg of cycloheximide (Sigma Chemical Co.) per ml.

RNA purification. Total cellular RNA was prepared by lysing cells in guanidinium thiocyanate solution (2) and centrifuging the lysate through a CsCl-EDTA cushion (6). Pelleted RNA was solubilized, extracted with phenol-chloroform, precipitated with ethanol, and solubilized in water. Cytoplasmic and nuclear RNAs were purified from cells fractionated by gentle vortexing in 0.5% Nonidet P-40, 10 mM Tris hydrochloride (pH 7.4), 10 mM NaCl, and 3 mM MgCl₂. The nuclear pellets and cytoplasmic supernatants were treated with sodium dodecyl sulfate-proteinase K, extracted with phenol-chloroform, and precipitated with ethanol.

In vitro-transcribed RNAs were generated with SP6 and T7 RNA polymerases with linearized proliferin cDNA

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cloned in pGEM vectors (Promega Biotec) used as templates. The construction of these recombinant plasmids has been described previously (27). Transcription reactions were carried out in the presence of [α - ^{35} S]UTP, and the RNA products were purified by phenol-chloroform extraction and precipitation with ethanol.

Hybridization analysis. Total cellular RNAs were electrophoresed on formaldehyde-agarose gels (12), transferred to nitrocellulose (26), and hybridized to ^{32} P-nick-translated (22) DNAs, including the proliferin cDNA clone PLF-2 (13), the *c-myc* clone pSVC-myc-1 (10), and a 1.5-kilobase (kb) proliferin intron fragment (unpublished data). Hybridizations were performed for 14 to 24 h at 42°C in 50% formamide, 5× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate), 4× Denhardt solution (1× Denhardt solution is 0.02% each bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 0.5% sodium dodecyl sulfate, 0.5% sodium pyrophosphate, 10 μ g of denatured salmon sperm DNA per ml, and 10⁶ dpm of probe per ml. Filters were rinsed in 2× SSC–0.1% sodium dodecyl sulfate at room temperature and then washed at 50°C for 1 to 2 h with several changes of 0.1× SSC–0.1% sodium dodecyl sulfate.

BALB/c 3T3 cultures in chamber slides were fixed in 4% paraformaldehyde in phosphate-buffered saline for 30 min at room temperature. Further treatment of the slides, including acetylation, hybridization to ^{35} S-RNA transcripts of proliferin (see above), and washing, was carried out as described by Harper et al. (8). After being washed, the slides were dehydrated by being rinsed in 30, 60, 90, and, finally, 100% ethanol (3 min per rinse). The slides were air dried, dipped into NTB-2 emulsion (Eastman Kodak Co.) diluted 1:1 with 0.6 M ammonium acetate, and exposed for 1 to 2 weeks. Slides were developed with Kodak D19 developer.

RESULTS

Dependence of proliferin expression on protein synthesis.

High levels of proliferin mRNA are not detected in BALB/c 3T3 cells first rendered quiescent and then stimulated with serum until several hours after serum stimulation (5). This delay in proliferin mRNA accumulation presumably reflects a dependence on events that occurred earlier, during release of cells from G₀. To test whether protein synthesis is essential for the accumulation of proliferin mRNA in serum-stimulated cells, quiescent BALB/c 3T3 cultures were stimulated with serum in the presence of 10 μ g of cycloheximide per ml. The addition of cycloheximide at this concentration resulted in more than 99% inhibition of protein synthesis, as determined by incorporation of [^{35} S]methionine into acid-insoluble counts (data not shown). The medium was supplemented with cycloheximide at the beginning of stimulation (0 h) or at 2, 4, or 6 h after stimulation was initiated. RNA was harvested from the cultures at 10 h poststimulation and analyzed for proliferin mRNA levels.

Quiescent BALB/c 3T3 cultures treated concurrently with cycloheximide and serum for 10 h did not express a detectable amount of proliferin mRNA (Fig. 1A). Delaying the addition of cycloheximide until after 2 or 4 h of serum treatment allowed the accumulation of proliferin mRNA, although at levels below those seen in the absence of the drug (approximately 50% of the untreated level, as estimated by densitometry). A higher level (80% of the untreated level) of proliferin mRNA accumulated if protein synthesis was inhibited for only the final 4 h of the 10 h-stimulation period. Thus, maximal proliferin gene expression was dependent on protein synthesis for at least the first 4 to 6 h of serum

FIG. 1. Effect of cycloheximide treatment on proliferin mRNA levels. Quiescent BALB/c 3T3 cell cultures were maintained in starvation medium (–) or stimulated with serum (+). The culture medium was supplemented with cycloheximide (CHX) (10 μ g/ml) either at the same time as that of serum addition (0) or at 2, 4, or 6 h after serum stimulation was initiated; a control serum-stimulated culture did not receive cycloheximide. All cultures were harvested for RNA 10 h after addition of serum. Electrophoretically separated RNAs were transferred to nitrocellulose and (A) hybridized to a proliferin probe and then (B) rehybridized to a *myc* probe. The positions of the 1-kb proliferin mRNA and 18S and 28S rRNAs are indicated.

stimulation. These RNAs were also hybridized to a probe for *c-myc* RNA (10), and as reported previously (9), *c-myc* RNA levels were superinduced by the addition of cycloheximide with serum to quiescent cells (Fig. 1B). After a 10-h exposure to serum, 3T3 cells still had elevated *myc* RNA levels, but this level was much higher in those cultures also treated with cycloheximide. The difference between *myc* and proliferin expression was especially clear in the culture treated with cycloheximide at the same time that serum was administered.

In all cases, the proliferin mRNA from the cycloheximide-treated samples displayed much less size heterogeneity than the untreated, stimulated sample. Hybridization of these RNAs to oligo(dT) and incubation with RNase H to destroy the poly(A) tails eliminated this heterogeneity and reduced the size of the proliferin-hybridizing RNA from all of these samples to the same length (data not shown).

Serum dependence of proliferin expression. The effect of cycloheximide on proliferin expression was evaluated in cell cultures exposed to high serum levels. It was not clear whether proliferin expression depended on both protein synthesis early in G₁ and a high serum concentration throughout G₁. To determine if maximal proliferin mRNA accumulation in late G₁ was dependent on the continuous presence of high serum levels, resting BALB/c 3T3 cells were stimulated with serum for various lengths of time, and then the medium was replaced with starvation medium. Ten hours after serum was initially added, cells were harvested and proliferin RNA levels were compared. The results (Fig. 2) demonstrate that some proliferin mRNA did accumulate in cells exposed to serum for only 1 h and then maintained in low-serum medium for 9 h. The level of proliferin mRNA increased if the serum treatment was extended to 2 h, and an even greater amount of proliferin mRNA was obtained with a 4-h serum stimulation. No difference in the amount of proliferin mRNA was seen among cultures stimulated for 4 h

FIG. 2. Effect on proliferin mRNA levels of variation in serum pulse duration. Quiescent BALB/c 3T3 cell cultures were fed with medium containing 20% fetal bovine serum for 0, 1, 2, 4, 6, or 10 h. After the serum pulse, the medium was replaced with starvation medium (0.5% serum) for the remainder of the 10-h period, when all cultures were harvested. Purified RNAs were analyzed by filter hybridization to a proliferin cDNA probe. The position of the 1-kb proliferin mRNA is indicated.

and starved for 6 h, stimulated for 6 h and starved for 4 h, or stimulated for 10 h without any subsequent starvation. These results demonstrate that the exposure of cells to serum for approximately 4 h is required along with protein synthesis for the accumulation of high levels of proliferin mRNA. In all samples, the proliferin mRNA appeared as a heterogeneous smear of approximately 1 kb, suggesting that the size distribution, unlike the final amount, of proliferin mRNA is independent of an extended exposure to serum in the medium.

Posttranscriptional control of proliferin gene expression. The rapid increase in amounts of certain mRNAs after release of cells from G_0 can be attributed to transcriptional activation of the corresponding genes (7). To determine whether proliferin gene transcription occurs in resting cells, total RNA was isolated from the cytoplasm and nuclei of resting and 12-h-serum-stimulated BALB/c 3T3 cells. These RNAs were hybridized to a 1.5-kb fragment from the intervening sequence between the second and third proliferin exons in a cloned proliferin gene (unpublished data). The results (Fig. 3A) indicate that incompletely processed proliferin transcripts are present in the nuclei of resting and stimulated cells, although at much lower levels in resting cells. This same pattern of high-molecular-weight RNAs is also seen with a proliferin cDNA probe (Fig. 2). Rehybridization of the Fig. 3A filter with the proliferin cDNA sequences detects the fully processed 1-kb mRNA in both the nuclear and cytoplasmic fractions of the stimulated cell culture (Fig. 3B). This 1-kb mRNA was not seen in either the nuclei or the cytoplasm of resting cells, indicating that some proliferin RNA processing events are lacking in resting cells or that the 1-kb mRNA is extremely unstable in resting cells.

The greater amount of precursor RNA detected in stimulated versus resting cells could be due to increased transcription. However, nuclear runoff experiments have not detected significant increases in proliferin transcription during serum stimulation (data not shown). Recently, we have

found that transcription from individual proliferin promoters in transfected cells is stimulated by serum (D. I. H. Linzer and J. C. Mordacq, submitted for publication), indicating that proliferin mRNA levels are controlled at both the transcriptional and posttranscriptional levels.

Heterogeneity of proliferin expression. The presence of low levels of proliferin transcripts in the resting-cell population could be due to high levels of expression in only a few cells. Proliferin expression was therefore investigated at the single cell level by in situ hybridization of resting and serum-stimulated cultures of BALB/c 3T3 cells to ^{35}S -labeled antisense RNA transcripts of proliferin cDNA. No hybridization was detected in any of the cells in the resting population (Fig. 4), but significant hybridization was found in the culture stimulated with serum for 12 h. The hybridization signal in the serum-stimulated culture varied from cell to cell; approximately one-third of the cells expressed high levels, one-third expressed moderate levels, and one-third expressed low or undetectable levels of proliferin mRNA. The amount of proliferin RNA detected per cell increased during the 12 h after serum stimulation and decreased to a low level by 24 h (Fig. 5). Cell-to-cell variation in the concentration of proliferin RNA was again evident; however, no strongly hybridizing cells were seen at 3 or at 6 h after stimulation. By 9 h poststimulation, a low percentage of cells showed moderate to high proliferin RNA concentrations. The maximum intensity per cell and the percentage of strongly hybridizing cells increased through the 12-h time point.

We have been able to rule out several possible explanations for this heterogeneity. Hybridizations of similar cultures with a probe for tubulin RNA yielded the same hybridization signal for each cell, indicating that the proliferin result is not an artifact of the hybridization protocol; the possibility that a significant percentage of cells were not responding to serum can be ruled out, since more than 95% of the stimulated cells incorporated $[^3\text{H}]$ thymidine during a 24-h labeling period; and well-separated colonies of 6 to 20 cells generated from single cells also yielded heterogeneous hybridization within the colony, demonstrating that

FIG. 3. Detection of proliferin transcripts in resting and stimulated cells. (A) RNA was purified from the nuclear (N) and cytoplasmic (C) fractions of resting (-) and 12-h-serum-stimulated (+) BALB/c 3T3 cells and hybridized to a proliferin-intron probe. Each lane contains 10 μg of total nuclear or cytoplasmic RNA. (B) The filter was rehybridized with a proliferin cDNA clone. The position of the 1-kb proliferin mRNA is indicated with an arrow. Size markers are from the RNA ladder from Bethesda Research Laboratories.

FIG. 4. Heterogeneity of proliferin mRNA levels in stimulated cell cultures. Resting (A) or 12-h-serum-stimulated (B) BALB/c 3T3 cultures were assayed for proliferin RNA levels by in situ hybridization to a single-stranded antisense proliferin probe. After hybridization and washing, the samples were treated with Kodak NTB-2 emulsion and exposed for 12 days.

the cell line itself is not heterogeneous (data not shown). Since proliferin protein is secreted (11a, 13, 19), it has the potential to act in *trans* on nonexpressing cells. We also considered the possibility that proliferin protein secreted from expressing cells down-regulates proliferin expression in neighboring cells; however, addition of purified proliferin protein (provided by Lee and Nathans) or conditioned medium from growing 3T3s to serum-stimulated BALB/c 3T3 cultures did not suppress the level of proliferin mRNA in the cell population (data not shown). Finally, serum-stimulated cultures were also pulse-labeled with [^{3} H]thymidine at various times during late G₁ and early S (a 1-h labeling period ranging from 10 to 17 h poststimulation). Serum-stimulated cells did not enter S phase with absolute synchrony (Fig. 6); a gradual increase in the percentage of cells in S phase was seen until, at 16 to 17 h poststimulation, 94% of the cells were labeled. At 12 h after stimulation, only 14 to 21% of the cell population had begun to synthesize DNA. If the time of proliferin expression is constant relative

to the time of entry into S phase for each cell, then this asynchrony could account for the observed heterogeneity in proliferin mRNA levels.

DISCUSSION

The accumulation of proliferin mRNA in BALB/c 3T3 cells in response to serum has been found to differ from the rapid induction of *fos* (4, 6), *myc* (9), and other mRNAs (3, 11) in several aspects. Proliferin mRNA was not detected immediately after serum stimulation, but in mid- to late G₁; new protein synthesis was required for the increase in proliferin mRNA levels; cells had to be maintained in a high serum concentration for several hours to generate maximal amounts of proliferin mRNA; and proliferin transcription occurred in resting cells, but these transcripts were not processed to stable 1-kb mRNA.

The addition to resting cells of cycloheximide with serum totally blocked the accumulation of proliferin mRNA. In contrast, *fos* (4), *myc* (9), and other serum-responsive RNAs (11) are superinduced in serum-stimulated cells treated with cycloheximide. Submaximal levels of proliferin mRNA were detected during late G₁ in cells treated with cycloheximide after 2 or 4 h of serum stimulation, while nearly maximal levels were obtained if the cycloheximide block was delayed until 6 h after serum addition. These results suggest that a protein (or proteins) that is essential for the accumulation of proliferin mRNA is synthesized early in G₁ and increases in concentration over several hours. Cycloheximide also alters the heterogeneity in proliferin mRNA poly(A) tail length, perhaps by depressing translation coupled poly(A) tail degradation or by inhibiting the synthesis of a protein factor that modifies proliferin mRNA.

The very early changes in gene expression after serum stimulation may be connected to the later appearance of proliferin mRNA. However, even though the levels of *c-fos* and other mRNAs have been shown to increase within the first hour after serum treatment, a 1-h pulse of serum did not result in high levels of proliferin mRNA in late G₁. Since a longer exposure of resting cells to serum was required for maximal proliferin mRNA levels, the early activation of these genes was insufficient by itself to drive proliferin expression. Coupled with the cycloheximide results, these data suggest that the protein synthesis essential for proliferin mRNA accumulation is not maintained upon serum withdrawal. After the first 4 to 6 h of serum stimulation, protein synthesis and serum exposure are no longer necessary for maximal proliferin mRNA production.

The serum response of several genes in 3T3 cells, including *fos* and *myc*, is due to transcriptional activation (7). However, posttranscriptional control appears to be the basis for increased *myc* RNA levels in the response of another fibroblast line to growth factors (1). Proliferin promoter activity is stimulated by serum in transfected cells (Linzer and Mordacq, submitted), but the regulation of proliferin mRNA accumulation also appears to have a posttranscriptional component, in either the processing or the stabilization of proliferin RNA. Even though no cytoplasmic (or fully processed nuclear) proliferin RNA was detected in resting BALB/c 3T3 cells, transcription of the proliferin genes was not completely dormant in these cells; high-molecular-weight RNA that hybridized to proliferin cDNA and to proliferin intron sequences was detected in the nuclear fraction of resting cells. It is unlikely that these results were due to a small percentage of the resting-cell population expressing high levels of proliferin mRNA, since no strongly

FIG. 5. Time course of proliferin expression. Resting BALB/c 3T3 cells were stimulated with serum for 3 (A), 6 (B), 9 (C), 12 (D), or 24 (E) h. The cells were hybridized in situ to a single-stranded antisense proliferin probe. The samples were washed and exposed to Kodak NTB-2 emulsion for 6 days. Photomicrographs were made by focusing above the plane of the cells to enhance the visualization of silver grains.

hybridizing cells were detected by in situ hybridization in a serum-starved culture. Thus, proliferin genes were being transcribed in resting cells, but mature proliferin mRNA was not being generated.

Edwards et al. (5) have reported that higher levels of proliferin (mitogen-regulated protein) RNAs are present in the nuclei of stimulated cells than in those of resting cells, and they attribute this difference to increased transcription. However, their analysis utilized slot-blotted RNA and could not detect different size classes of proliferin (mitogen-regulated protein) RNAs. We also found an increase in nuclear proliferin RNA levels after serum stimulation, some of which was due to the accumulation of 1-kb mRNA in stimulated cells, while no fully processed proliferin RNA

was detected in resting-cell nuclei. Multiple proliferin genes are present in the mouse genome, and at least two of these genes are expressed (27). Hybridization signals may therefore reflect a complex average of transcription from functional genes and pseudogenes or from genes with various regulatory programs. It is conceivable, therefore, that the observed transcripts in resting cells could not be processed to mRNA and that the proliferin mRNA detected after serum stimulation was derived from other gene copies.

One possible explanation for the increasing amount of proliferin RNA detected in cell cultures during the 12 h after serum stimulation (14) is that a subset of the population rapidly and transiently accumulated high levels of this RNA and that the number of expressing cells increased during G_1 .

FIG. 6. Asynchronous entry into S phase of serum-stimulated cells. Quiescent BALB/c 3T3 cells were stimulated with serum and pulse-labeled with [^{3} H]thymidine for 1 h at times ranging from 10 to 17 h after serum addition. Labeled cells were detected with Kodak NTB-2 emulsion, and the percentage of labeled nuclei was determined from approximately 400 cells at each time point.

Alternatively, the amount of proliferin RNA per cell could increase from the time of stimulation to that of entry into S phase. Only low proliferin RNA levels were detected by *in situ* hybridization in cells stimulated with serum for 3 or 6 h. A low percentage of cells had high levels of this RNA by 9 h poststimulation, but both the number of highly expressing cells and the amount of proliferin RNA per cell continued to increase through 12 h after stimulation. Thus, stimulated cells appeared to accumulate proliferin RNA throughout G_1 , with this RNA attaining a high concentration per cell only in late G_1 . However, even in late G_1 , cellular levels of proliferin RNA were heterogeneous in the population. This heterogeneity can be attributed largely to asynchrony in the rate of transit of serum-stimulated cells through G_1 , a potential problem for the analysis of other genes that respond to serum with delayed kinetics. The appearance of high levels of proliferin in late G_1 correlates with the time that DNA synthesis is first detected in the population. The observations that proliferin RNA levels were heterogeneous during late G_1 and that serum-stimulated cells entered S phase asynchronously also raise the possibility that a connection exists between the time of maximal proliferin expression and the onset of cellular DNA synthesis.

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