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Platelet-Derived Growth Factor-Modulated Translatable mRNAs

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The treatment of density-arrested BALB/c 3T3 cells with electrophoretically homogeneous or highly purified preparations of the platelet-derived growth factor (PDGF) stimulated the rapid and selective accumulation of several species of abundant mRNA identified by cell-free translation. These translatable mRNAs appeared long before entry into the S phase. Less PDGF was required for selective mRNA accumulation than for PDGF-modulated DNA synthesis. The translatable mRNAs also accumulated after addition of the epidermal growth factor but not after addition of insulin or platelet-poor plasma. Their selective accumulation was blocked by addition of actinomycin D. Three classes of PDGF-modulated mRNAs were defined. An early (primary) RNA appeared within 30 to 60 min of PDGF addition; its accumulation was not blocked by cycloheximide. Another early mRNA also appeared within 60 min, but treatment with both PDGF and cycloheximide was required for optimal accumulation. A third class, secondary RNAs, began to accumulate later at 90 to 120 min; the appearance of this class was inhibited by cycloheximide. One- and two-dimensional gel electrophoresis of translation products demonstrated that a spontaneously transformed BALB/c 3T3 (ST2-3T3) cell line, which does not require PDGF or epidermal growth factor for growth, constitutively accumulated the secondary growth factor-regulated mRNAs. The accumulation of these translatable mRNAs may be required for PDGF-modulated DNA synthesis.

The replication of mammalian connective tissue cells such as the mouse BALB/c 3T3 cell line is regulated by polypeptide growth factors present in serum (3). The addition of whole serum or purified serum growth factors to quiescent cells stimulates a pleiotypic response, characterized by increased transport of nutrients and increased macromolecular synthesis (20). These early G_0/G_1 events culminate in DNA synthesis and mitosis. Many elements of the pleiotypic response have been dissociated from DNA synthesis; however, protein synthesis is essential (6, 7, 31).

A brief treatment with the platelet-derived growth factor (PDGF) (35, 40), a family of cationic proteins (1, 2, 11, 17, 33) present in serum, initiates the replicative response by stimulating density-arrested BALB/c 3T3 cells to become "competent" to synthesize DNA (30, 41). The addition of platelet-poor plasma, which lacks PDGF (42), allows these cells (but not incompetent cells) to enter the S phase (30, 31). Plasma can be replaced by the epidermal growth factor (EGF) and insulin to allow a single round of DNA synthesis (37). Virus-transformed (39), chemical-transformed (36), or spontaneously transformed (36, 37) BALB/c 3T3 cells do not require PDGF for growth and can replicate in medium supplemented with plasma alone.

Either whole serum (44) or PDGF (28, 29, 36) rapidly stimulates quiescent BALB/c 3T3 cells to synthesize proteins in a selective fashion, whereas plasma does not. Such selective synthesis may be required for the acquisition of competence because a spontaneously transformed tumorigenic variant of BALB/c 3T3 cells (ST2-3T3), which requires neither PDGF nor EGF for growth (37), synthesizes the PDGF-modulated proteins constitutively (28, 36, 37). Both the acquisition of PDGF-induced competence (43) and the selective synthesis of these proteins (28, 36) are blocked by inhibitors of RNA synthesis, suggesting that both processes are regulated at a transcriptional level. However, there is no direct evidence that PDGF regulates the accumulation of translatable mRNA. We used in vitro mRNA

translation to identify and quantify growth factor-modulated mRNAs. We found that either PDGF or EGF stimulated BALB/c 3T3 cells to accumulate several sets of mRNAs in an orderly fashion. The accumulation of primary mRNA began within 30 min of PDGF addition and did not require protein synthesis. Secondary mRNAs appeared some time later, but long before cellular entry into the S phase. The accumulation of these secondary mRNAs required protein synthesis, a finding which suggests that the primary gene product(s) may regulate the appearance of the secondary ones. Because the spontaneously transformed ST2-3T3 cell line, which does not require PDGF or EGF for growth, constitutively accumulates both sets of mRNAs, the expression of these gene products may regulate, in part, both the normal and abnormal growth response.

(A portion of this work has been presented elsewhere in preliminary form [37].)

MATERIALS AND METHODS

Cell-free translation. BALB/c 3T3 (clone A31) or clonal ST2-3T3 (clone 2) (36) cells were grown in Dulbecco modified Eagle medium. Cells were grown to confluence on 21-cm^2 dishes and used 4 days after a change of medium. The cultures were either treated with preparations of PDGF, EGF, insulin, or plasma or left untreated. As indicated, the medium was removed, and the cultures were washed twice with phosphate-buffered saline at 4°C. Each plate was scraped with a rubber policeman into 2 ml of lysis buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-hydrochloride [pH 7.9], 0.65% Nonidet P-40) at 4°C. The nuclei were pelleted in a microfuge at 4°C for 2 min, and the supernatants were brought to 0.5% sodium dodecyl sulfate (SDS)–1 mM EDTA–0.25 M NaCl and extracted three times with a 1:1 mixture of water-saturated phenol-chloroform containing 2% 8-hydroxy-quinoline and once with chloroform. The RNA was ethanol precipitated twice from 400 mM potassium acetate, dried in vacuo, and dissolved in 20 to 100 μl of water, and its concentration was determined by absorbance at 260 nm. A 2-μg sample was translated in micrococcal nuclease-treated reticulocyte lysates (27) containing [^{35}S]methionine, as described by Braell and Lodish (5), but RNase inhibitors were not used. After 60 min, the incubation mixtures were cooled to 0°C, and 1-μl volumes were transferred in triplicate to disks of filter paper (3MM; Whatman, Inc., Clifton, N.J.), washed twice with 5% trichloroacetic acid at 80°C, and treated by ethanol and air drying for scintillation counting to determine the incorporation of [^{35}S]methionine into protein. The amount of protein synthesized was proportional to the amount of RNA added after the addition of 0.5 to 5.0 μg of cytoplasmic RNA.

One-dimensional gel electrophoresis. A total of 50,000 cpm of trichloroacetic acid-insoluble material from each reaction mixture was diluted into sample buffer (62.5 mM Tris-hydrochloride [pH 6.8], 1% SDS, 10% 2-mercaptoethanol, 10% glycerol, 0.0005% bromophenol blue), heated to 100°C for 2 min, and applied to Laemmli (22) slab gels containing 10% polyacrylamide. After electrophoresis, the gels were fixed in 15% trichloroacetic acid and processed for fluorography. The gels were overlaid with prefogged XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) and not fully exposed to ensure that the optical absorbance of each visible band was proportional to the amount of each translatable product (23). In some experiments, autoradiograms were scanned by a densitometer with an integrating device (Quick Scan, Helena Laboratories, Beaumont,Tex.), and the absorbance of individual protein bands was determined. The relative absorbance of each band was then calculated by dividing the absorbance of the PDGF-modulated band by the absorbance of that band elicited by RNA from untreated control cells. The exact amount of absorbance of individual [^{35}S]methionine-labeled PDGF-induced protein bands varied somewhat from experiment to experiment possibly because of variations in the intensity of the control bands or because of variations in the sensitivity of the cells; however, the intensity of the PDGF-modulated bands was always greater than that of the untreated controls. The molecular weights of the translation products were estimated from the position of known ^{14}C-labeled protein molecular-weight standards (Bethesda Research Laboratories, Gaithersburg, Md.). Translation products with an estimated molecular weight of 125,000 or more were noted in all experiments.

Two-dimensional gel electrophoresis. Two-dimensional non-equilibrium gel electrophoresis was performed by a modification of the technique of O'Farrell et al. (26). Approximately 5×10^5 cpm of acid-insoluble material was added to the electrofocusing sample buffer (9.5 M urea, 2% Nonidet P-40, 5% 2-mercaptoethanol, 1.6% Ampholines [pH 5 to 7; LKB Instruments, Inc., Rockville, Md.], 0.4% Ampholines [pH 3.5 to 10; LKB Instruments, Inc.]) and clarified by centrifugation at 1,400 × *g* for 5 min before application to the pH gradient gels. After completion, the pH gradient gels were equilibrated with SDS electrophoresis buffer and run on a second dimension on a 10% polyacrylamide gel together with molecular-weight markers.

Growth factors and metabolic inhibitors. Most experiments were done with human PDGF purified 2,000-fold through the Bio-Gel P150 (Bio-Rad Laboratories, Richmond, Calif.) stage of our protocol (2). This PDGF had an activity of 6.7×10^4 U/mg. Some experiments were done with electrophoretically homogeneous PDGF (2) which was obtained by employing two additional high-pressure liquid chromatography steps, reverse-phase chromatography and molecular sieving. Continuous (36-h) treatment with pure PDGF (0.5 to 1.0 ng/ml) promoted DNA synthesis. This PDGF appeared as a single band with a molecular weight of 33,000. Mouse EGF (8) was obtained from Collaborative Research, Inc., Waltham, Mass., and porcine insulin was purchased from Sigma Chemical Co., St. Louis, Mo. Human platelet-poor plasma was prepared as described (30). Because growth factors adhere nonspecifically to plastic surfaces, the PDGF, EGF, and insulin were added to cells in the presence of 0.3% platelet-poor plasma. Actinomycin D and cycloheximide (Sigma) were used at a concentration of 5 μg/ml; the additions inhibited >95% of RNA and protein synthesis, respectively.

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DNA synthesis. Because there is some variation in the sensitivity of BALB/c 3T3 cells to both PDGF-induced mRNA accumulation and DNA synthesis, assays for mRNA accumulation and DNA synthesis were done at the same time with duplicate cultures. To measure DNA synthesis, confluent BALB/c 3T3 cell cultures on 21-cm^2 plates were treated with PDGF for various lengths of time and then transferred to medium supplemented with an optimal concentration of plasma. For 21-cm^2 (60-mm) plates, on which these experiments were performed, this concentration was 30%. The medium was supplemented with [^{3}H]thymidine (10 μCi/ml, 6.7 Ci/mmol). After 30 h, the cells were fixed and processed for autoradiography. The nuclei from at least 200 contiguous cells from a representative section were counted in each culture.

RESULTS

PDGF-modulated mRNA. To study mRNA accumulation, cultures of density-arrested BALB/c 3T3 cells were treated with PDGF purified through the Bio-Gel P150 step of our protocol. Cultures were harvested at 1 or 6 h after PDGF addition; as a control, a duplicate culture was not treated with PDGF. Cytoplasmic RNA was prepared for translation in an mRNA-dependent reticulocyte lysate system. The [^{35}S]methionine-labeled proteins were analyzed by polyacrylamide gel electrophoresis and detected by fluorography. Proteins that are synthesized in vitro and quantified in this manner provide a measure of the accumulation of distinct species of translatable mRNA. RNA prepared from BALB/c 3T3 cells treated with PDGF for 1 h directed the preferential synthesis of tpI, a 29,000-dalton translation product (Fig. 1A). RNA from cultures treated with PDGF for 6 h did not stimulate tpI synthesis, but two other translation products, tpII (35,000 daltons) and tpIII (55,000 daltons), were prominent (Fig. 1B). Additional putative PDGF-modulated translation products were occasionally noted in the reaction mixtures directed by RNA from PDGF-treated cultures, but were not consistently found.

Temporal sequence of translatable mRNA accumulation. To define early- and late-appearing sets of PDGF-modulated mRNA, the accumulation of these three mRNAs was determined as a function of time. Density-arrested BALB/c 3T3 cell cultures were treated with Bio-Gel PDGF for various lengths of time before cytoplasmic RNA was harvested for in vitro translation. The translation products were analyzed by fluorography after one-dimensional gel electrophoresis. The tpI, tpII, and tpIII bands were scanned with a densitometer to quantify the amount synthesized in vitro. The optical absorbance of the band for each translation product is a function of the amount of each species of translatable mRNA present. Quantification of PDGF-regulated translatable mRNAs was relative to that of the mRNAs of untreated cells. The accumulation of tpI mRNA began within 30 min of PDGF addition and reached a maximum at 60 (Fig. 2A) to 90 min (data not shown); the level of tpI mRNA then decreased rapidly and by 240 min was only slightly greater than that of untreated cultures. By contrast, the increased accumulation of tpII (Fig. 2B) and tpIII (Fig. 2C) was first detected at 240 and 90 min after PDGF addition, respectively. Both of these RNA species continued to show increased accumulation for 360 min of the treatment. These results show that individual mRNA species accumulate after different lag periods and serve to define early (tpI) and late (tpII and tpIII) PDGF-modulated mRNAs.

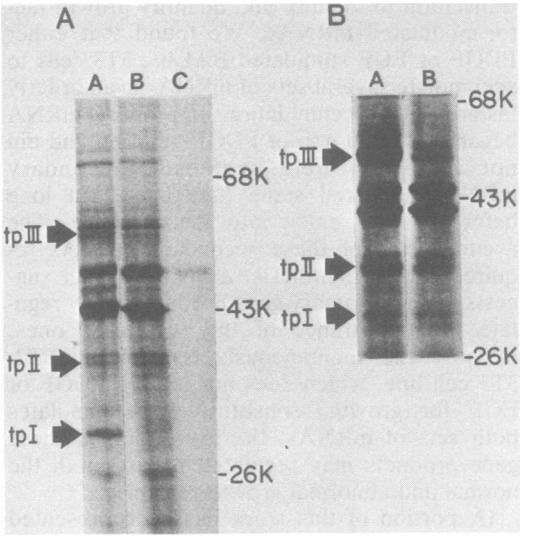

FIG. 1. PDGF modulates the selective increase of distinct species of translatable mRNA. (A) Density-arrested BALB/c 3T3 cells were treated with Bio-Gel PDGF (3 μg/ml) for 1 h (lane A) or left untreated in PDGF-depleted medium (38) (lane B) before cytoplasmic RNA was harvested for translation in micrococcal nuclease-treated reticulocyte lysates. Lane C depicts the translation products of the reticulocyte lysates without the addition of BALB/c 3T3 cell cytoplasmic RNA. For lanes A and B, 50,000 cpm of acid-insoluble material was applied to the gel. For lane C a quantity (3 μl) equal to the mean of the volumes applied to lanes A and B was used. (B) Density-arrested BALB/c 3T3 cultures were treated with Bio-Gel PDGF (17 μg/ml) for 6 h (lane A) or left untreated (lane B) before cytoplasmic RNA was harvested for in vitro translation. The positions of tpI, tpII, and tpIII and molecular weight markers are shown.

Effect of pure PDGF and other growth factors on translatable mRNA. Density-arrested BALB/c 3T3 cells were treated with electropho-

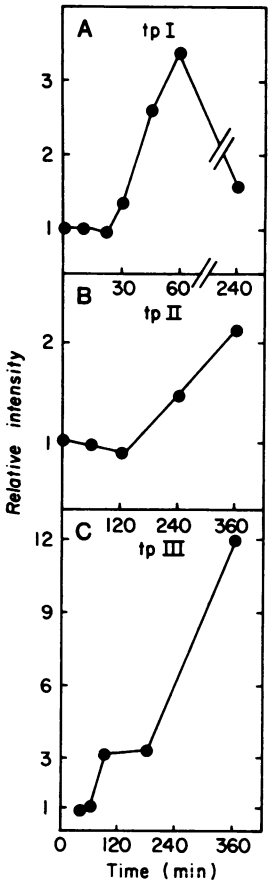

FIG. 2. The sequential accumulation of PDGF-modulated translatable mRNAs. Density-arrested BALB/c 3T3 cells were treated with Bio-Gel PDGF (3 μg/ml) for the times indicated. Cytoplasmic RNA was then prepared, translated, and analyzed by gel electrophoresis. The densities of the tpI, tpII, and tpIII bands were quantitated by scanning the fluorograms with a densitometer and integrating the area under the curves. The densities of both the tpI (A) and the tpII (B) bands at various times are relative to untreated cultures at zero time. The density of tpIII (C) is relative to cultures treated with 30% plasma. Plasma does not stimulate the accumulation of tpI, tpII, or tpIII mRNA as compared with untreated cultures (Table 1). There was less than a 10% variation in the densities of tpI, tpII, and tpIII with time in plasma-treated or untreated cultures.

retically homogeneous PDGF (25 ng/ml) or left untreated for 6 h before cytoplasmic RNA was harvested for in vitro translation. The relative synthesis of tpI, which is directed by an early mRNA, and tpIII, directed by a late mRNA, was analyzed. The pure PDGF stimulated a 2.3-fold increase in tpIII and a 1.7-fold increase in tpI synthesis in these cultures harvested at 5 h (Table 1), demonstrating that the appropriate mRNAs accumulate in response to PDGF. The latter number represents a minimal figure because optimal accumulation of tpI mRNA occured at 1 h (Fig. 2). In all other experiments, tpI and tpIII mRNAs were analyzed from cultures harvested 1 and 6 h after growth factor addition, respectively.

EGF (8) at 10 to 100 ng/ml stimulated a three- to four-fold increase in both tpI and tpIII mRNA accumulation (Table 1). This limited study also showed that the EGF-modulated mRNAs had a time dependency reminiscent of the PDGF-modulated mRNAs; for both growth factors tpI mRNA accumulated before tpIII mRNA. In contrast to PDGF and EGF, neither insulin nor plasma had any appreciable effect. Thus, the accumulation of these translatable mRNAs is specific for PDGF and EGF.

Relationship to DNA synthesis. Duplicate cultures of BALB/c 3T3 cells were treated with various concentrations of Bio-Gel PDGF, and both DNA synthesis and the preferential accumulation of translatable mRNAs were examined. One group was treated with PDGF for 1 or 6 h to quantify tpI or tpIII mRNAs, respectively. A second group was treated with PDGF for the same lengths of time and then transferred to medium containing an optimal concentration of plasma and [^{3}H]thymidine to quantitate the percentage of cells rendered competent to synthesize DNA. The accumulations of both tpI and tpIII mRNAs exhibited similar PDGF concentration dependence (Fig. 3); treatment with as little as 0.1 μg of Bio-Gel PDGF per ml stimulated increased accumulation of translatable tpI and tpIII mRNAs. However, a 6-h treatment with this concentration of PDGF had little, if any, effect on DNA synthesis (Fig. 3B). A 1- or 6-h treatment with 1 μg of Bio-Gel PDGF per ml stimulated maximal accumulation of both tpI and tpIII mRNAs. However, this concentration induced only 20% (1 h) or 60% (6 h) of the cells to enter the S phase (Fig. 3A and B). Treatment with 3 μg/ml increased the percentage of cells that entered the S phase whether they had been treated with PDGF for 1 or 6 h. Thus, less PDGF is required for tpI or tpIII mRNA accumulation than for DNA synthesis.

Requirement for protein synthesis. Density-arrested BALB/c 3T3 cells were treated with both PDGF and cycloheximide to determine whether protein synthesis is required for accumulation of the PDGF-modulated mRNAs. A 1-h treatment with Bio-Gel PDGF alone caused the increased accumulation of tpI mRNA as compared with untreated cultures (Fig. 4A, lanes A and B). The addition of cycloheximide to the PDGF-treated cultures did not prevent the accumulation of tpI mRNA (lane C); RNA from these cultures directed the preferential synthesis of an additional polypeptide (tpIV) with a 32,000

molecular weight (lane C). The tpIV mRNA was present in cultures treated with PDGF (lane B) or cycloheximide (lane D) alone, but at much reduced levels. In the presence of both PDGF and cycloheximide, accumulation of tpIV mRNA approached that of PDGF-modulated tpI mRNA. Thus, protein synthesis is not required for the appearance of tpI and tpIV mRNAs, which accumulate within 1 h or less of PDGF addition. The addition of actinomycin D, however, completely inhibited the PDGF-modulated accumulation of these mRNAs (lanes E and F), suggesting that such accumulation may be regulated, in part, at a transcriptional level.

A 4-h treatment with PDGF resulted in an increase in translatable tpII and tpIII mRNA accumulation and a decrease in tpI mRNA (Fig. 4B, lanes A and B). The addition of cycloheximide to the PDGF-treated culture prevented the accumulation of both the tpII and tpIII mRNAs (lane C). Furthermore, cycloheximide treatment inhibited the normal decrease in tpI mRNA levels which usually occurred within 4 h of PDGF addition; however, tpIV mRNA was not detected in these cultures. These results indicate that protein synthesis is required for the accumulation of tpII and tpIII mRNAs, which appear late after PDGF addition. Furthermore, inhibition of protein synthesis blocks the normal decrease in accumulation of some species of early PDGF-modulated mRNAs.

Constitutive accumulation. To evaluate the functional significance of these growth factor-modulated mRNAs, the intracellular levels of translatable RNAs were analyzed in spontaneously transformed ST2-3T3 cells. Although ST2-3T3 cells replicate to a sixfold higher saturation density than BALB/c 3T3 cells, they become growth arrested at confluence with a G_1 DNA content (36). Unlike the parent BALB/c 3T3 cell line, the addition of PDGF to density-arrested ST2-3T3 cells has little if any effect on either DNA or preferential protein synthesis (28, 36). Density-arrested ST2-3T3 cells were either treated with Bio-Gel PDGF for 6 h or left untreated before the isolation of cytoplasmic RNA for translation and analysis by one-dimensional gel electrophoresis. In contrast to BALB/c 3T3 cells, PDGF had no effect on the intracellular concentration of tpI, tpII, or tpIII mRNAs (Fig. 5). Because both tpII and tpIII mRNAs of PDGF-treated BALB/c 3T3 cells maximally accumulate at 6 h, this experiment demonstrated that these two late mRNAs constitutively accumulate in ST2-3T3 cells. These mRNAs accumulate in the absence of PDGF.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was used to demonstrate that the translatable mRNAs which constitutively accumulate in ST2-3T3 cells direct the synthesis of the same translation products as the PDGF-modulated mRNAs of BALB/c 3T3 cells. BALB/c 3T3 cells were treated with Bio-Gel PDGF or platelet-poor plasma for 6 h before mRNA was harvested for translation. Two-dimensional gel electrophoresis allowed the identification of major PDGF-modulated spots corresponding to tpI, tpII, and tpIII (Fig. 6A and B). In the PDGF-treated cells, the late translation products (tpII and tpIII) were prominent. Of note was the finding that the prominent tpIII spot increased ca. 50-fold in response to PDGF. The use of two-dimensional gel electrophoresis allowed the recognition of this substantial increase in tpIII mRNA because it isolated this translation product from others of similar molecular weight.

RNA was also harvested from density-arrested ST2-3T3 cells after treatment with platelet-poor plasma for 6 h. Two-dimensional gel elec-

TABLE 1. Effects of growth factors on translatable mRNA accumulation

Growth factor	Concn	Translatable mRNA accumulation[a] of:	
		tpI	tpIII
Pure PDGF	25 ng/ml	170[b]	230
EGF	0.1 ng/ml	120	140
	1 ng/ml	220	170
	10 ng/ml	300	350
	100 ng/ml	200	375
Insulin	6.0 μg/ml	110	100
Plasma	30%	100	100
Untreated		100	100

[a] The medium was removed from confluent BALB/c 3T3 cells and replaced with fresh Dulbecco modified Eagle medium containing 0.3% plasma and the growth factors as indicated. The cultures were harvested to prepare cytoplasmic RNA for in vitro translation. Cultures were harvested at 1 h to analyze tpI mRNA accumulation and at 6 h to analyze tpIII mRNA accumulation. After translation, 50,000 cpm of the acid-insoluble translation products was displayed on one-dimensional SDS-gels; the fluorograms were scanned with a densitometer to quantify tpI and tpIII. The density of each translation product is a function of the accumulation of each species of translatable mRNA. The density of each translation product is given as the percent of that product for untreated cultures which were not transferred to growth factor-supplemented medium. This table is the composite of several experiments, each of which had an untreated control culture normalized to 100%.

[b] The percentage of tpI mRNA for pure PDGF was determined from cultures harvested at 5 h, which is beyond the optimal time for tpI mRNA accumulation.

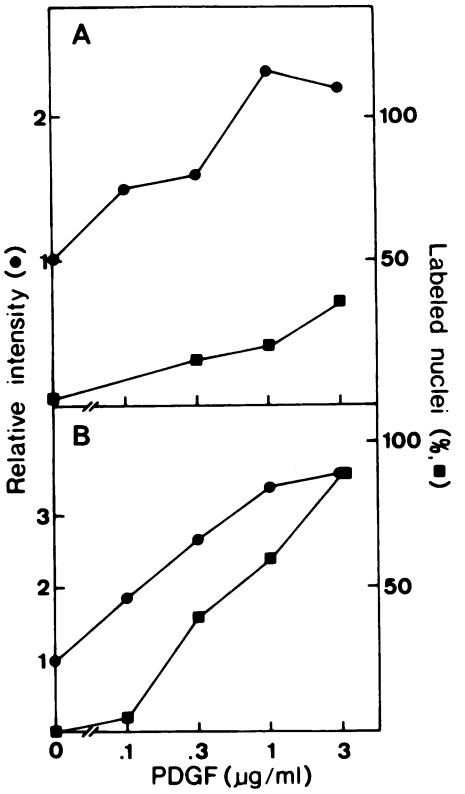

FIG. 3. Less PDGF was required for stimulation of tpI and tpIII mRNA than for PDGF-modulated competence. Density-arrested BALB/c 3T3 cells were treated with various concentrations of Bio-Gel PDGF for 1 (A) or 6 (B) h. The RNA was harvested from one group of cultures (●) and translated, and the amount of tpI (A) or tpIII (B) relative to cultures treated with 30% plasma was determined. A duplicate group of cultures (■) was transferred to medium supplemented with 30% plasma containing [^{3}H]thymidine. After 30 h the cells were fixed and processed for autoradiography to determine the percentage of cells that synthesized DNA (30).

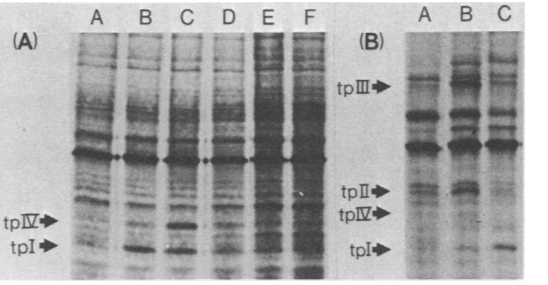

FIG. 4. Requirement for RNA and protein synthesis. Density-arrested BALB/c 3T3 cells were treated with cycloheximide (5 μg/ml) or actinomycin D (5 μg/ml), or left untreated for 30 min. The cultures were transferred to fresh medium containing the same concentration of inhibitor and 0.3% plasma with or without Bio-Gel PDGF (3 μg/ml). (A) At 1 h after addition of PDGF the cultures were harvested, and cytoplasmic RNA was processed for translation and SDS-gel electrophoresis. Lane A, untreated; lane B, PDGF; lane C, PDGF plus cycloheximide; lane D, cycloheximide; lane E, PDGF plus actinomycin D; lane F, actinomycin D. (B) At 4 h after addition of PDGF the cultures were harvested for translation. Lane A, untreated; lane B, PDGF; lane C, PDGF plus cycloheximide. The positions of tpI through tpIV are shown. The experiment in Fig. 1A was performed at the same time as that shown in Fig. 4B. The untreated controls were the same. Separate figures are used for clarity.

trophoresis of the ST2-3T3 cell translation products revealed a close resemblance to the translation products of the PDGF-treated BALB/c 3T3 cells (Fig. 6C). Compared to plasma-treated BALB/c 3T3 cells, there was an increase in the optical absorbance of the tpI and tpII spots. Furthermore, there was approximately a 50-fold increase in the prominent tpIII spot compared with plasma-treated BALB/c 3T3 cells. Density-arrested ST2-3T3 cells that were not treated with plasma displayed a similar pattern (data not shown). The data indicate that ST2-3T3 cells constitutively accumulate the secondary PDGF-modulated mRNAs of BALB/c 3T3 cells.

DISCUSSION

PDGF stimulated the selective accumulation of several species of translatable mRNA in density-arrested BALB/c 3T3 cells. These mRNAs appeared to be abundant because their translation products could be readily recognized on one- or two-dimensional gels. The PDGF-modu-

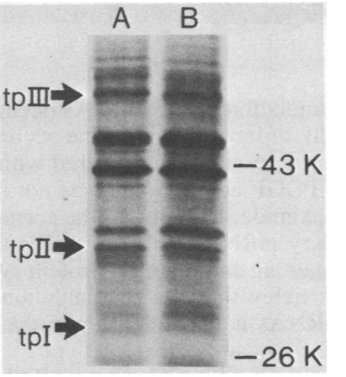

FIG. 5. Constitutive accumulation of translatable tpII and tpIII mRNAs by ST2-3T3 cells. Density-arrested ST2-3T3 cultures were treated with (A) Bio-Gel PDGF (3 μg/ml) for 6 h or (B) left untreated, and cytoplasmic RNA was prepared for translation. The translation products were resolved by SDS-gel electrophoresis. The positions of tpI, tpII, and tpIII are indicated.

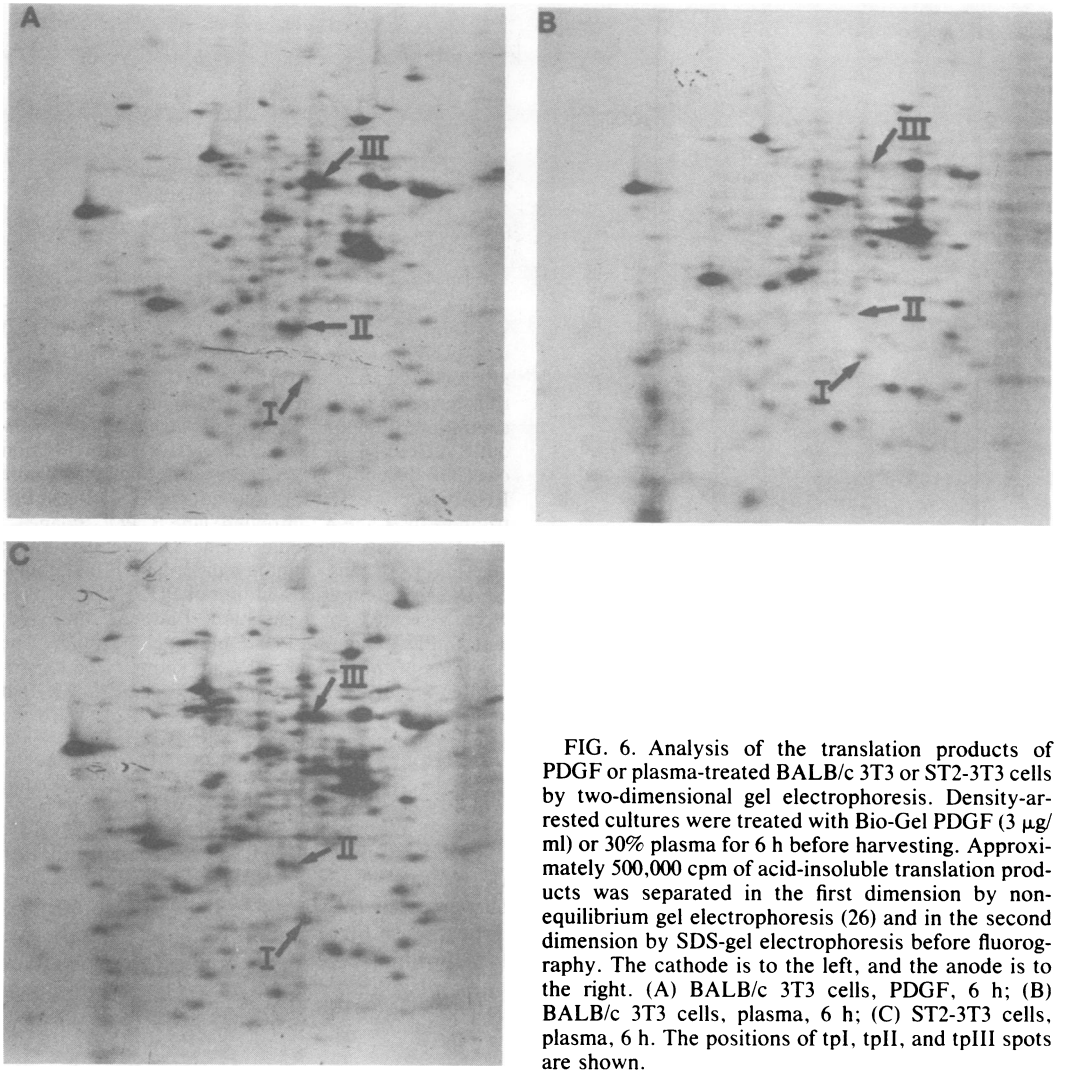

FIG. 6. Analysis of the translation products of PDGF or plasma-treated BALB/c 3T3 or ST2-3T3 cells by two-dimensional gel electrophoresis. Density-arrested cultures were treated with Bio-Gel PDGF (3 μg/ml) or 30% plasma for 6 h before harvesting. Approximately 500,000 cpm of acid-insoluble translation products was separated in the first dimension by nonequilibrium gel electrophoresis (26) and in the second dimension by SDS-gel electrophoresis before fluorography. The cathode is to the left, and the anode is to the right. (A) BALB/c 3T3 cells, PDGF, 6 h; (B) BALB/c 3T3 cells, plasma, 6 h; (C) ST2-3T3 cells, plasma, 6 h. The positions of tpI, tpII, and tpIII spots are shown.

lated accumulation of the mRNAs occurred in a sequentially ordered fashion. The accumulation of tpI, a primary mRNA, occurred within 30 to 60 min of PDGF addition and was not inhibited by cycloheximide. In contrast, the accumulation of secondary mRNAs (tpII and tpIII) occurred after a longer lag and required protein synthesis. It appears likley that the accumulation of secondary mRNAs is dependent upon the translation of the primary species.

Previous studies (28, 36) of intact BALB/c 3T3 cells demonstrated a specificity in growth factor-modulated protein synthesis. EGF induced many of the same proteins as PDGF, but high concentrations (100 ng/ml) were required. The present study used cell-free translation to analyze the expression of growth factor-modulated gene products. This system allows the identification of proteins which might not be readily detectable in whole cells because of post-translational modification or rapid protein turnover. It appears that PDGF and EGF stimulate the selective accumulation of similar translatable mRNAs. Furthermore, both PDGF and EGF rapidly cause tyrosine-specific phosphorylation of the same proteins of Swiss 3T3 cells within minutes of addition (9). One possibility is that tyrosine-specific phosphorylation is required for the preferential accumulation of the translatable mRNAs. There is a degree of growth factor specificity in the stimulation of these translatable mRNAs because neither insulin nor platelet-poor plasma stimulates their accumulation. Human plasma contains EGF, but at concentrations (<1 ng/ml [10]) which appeared to be too low (Table 1) to stimulate the selective accumu-

lation of these translatable mRNAs. Plasma, however, provides an appropriate concentration of growth factors to allow PDGF-treated cells to synthesize DNA (30).

Several lines of evidence suggest that PDGF-modulated mRNAs may regulate, at least in part, PDGF-induced mitogenesis. Both the PDGF-induced mitogenic response (43) and the accumulation of primary mRNA are blocked by inhibitors of RNA synthesis. Furthermore, spontaneously transformed ST2-3T3 cells, which do not require PDGF (or EGF) for growth (37), constitutively accumulate the secondary PDGF-modulated mRNAs. ST2-3T3 cells, as well as other spontaneously and carcinogen-transformed BALB/c 3T3 cells which have lost the PDGF growth requirement, constitutively synthesize (36) a 35,000-dalton major excreted protein (14). PDGF rapidly stimulates responsive nontransformed BALB/c 3T3 cells to increase their synthesis of the major excreted protein (36). Taken together, these results are consistent with the hypothesis that PDGF-modulated proteins play a role in regulating the growth of nontransformed BALB/c 3T3 cells. Constitutive accumulation of PDGF-modulated gene products could provide a mechanism for the loss of cellular growth control that typifies transformed cells. It is, of course, possible that these PDGF-modulated gene products are part of the pleiotypic response (20), but have a non-growth-related function.

The identification of the PDGF-modulated translation products requires further study. One of these products (tpII) has a molecular weight of 35,000, which is similar to that of PDGF-modulated major excreted protein synthesized by intact cells. However, the major excreted protein is a phosphorylated glycoprotein (15) which undergoes post-translational modification; it has a molecular weight of 33,000 in a cell-free translation system (16).

PDGF appears to be representative of a recently recognized group of polypeptide hormones which rapidly stimulate the accumulation of specific species of cytoplasmic mRNA. Prolactin mRNA accumulates in the GH_4 rat pituitary cell line in response to either thyrotropin-releasing hormone (32) or EGF (24). Furthermore, EGF stimulates AKR-2B mouse cells to accumulate mRNA related to a class of retrovirus-like sequences (13). The present study demonstrated that PDGF and EGF regulate the sequential appearance of several distinct species of mRNA.

The transcription of certain gene products is regulated as cells traverse the growth cycle. The transcription and accumulation of both dihydrofolate reductase mRNA (19) and histone mRNA (34) often increase as cells approach and enter the S phase. Both of these proteins are needed for DNA synthesis. In contrast, the increased expression of growth factor-modulated mRNAs is not a result of cells traversing the growth cycle. PDGF-treated BALB/c 3T3 cells require treatment with growth factors in plasma to progress toward the S phase (30, 31). Furthermore, growth-arrested (36, 37) ST2-3T3 cells constitutively accumulate these mRNAs. It appears that the accumulation of these mRNAs may be required for growth factor action, rather than being a result of cell cycle traverse.

Cycloheximide inhibited the accumulation of PDGF-modulated secondary mRNAs and also altered the primary mRNA response. The addition of cycloheximide to PDGF-treated BALB/c 3T3 cells also inhibited the normal decrease in translatable tpI mRNA, which occurs even in the presence of PDGF. Such treatment also stimulated the accumulation of tpIV mRNA, a PDGF-modulated species which could not be recognized in the absence of this inhibitor. The optimal accumulation of this translatable early mRNA required the addition of both PDGF and cycloheximide.

Several of the early events in PDGF-induced mitogenesis have been defined. PDGF binds to a specific saturable high-affinity membrane receptor (4, 18, 41, 42) and induces tyrosine-specific phosphorylation of membrane (12, 25) and cytoplasmic (9) proteins. However, the mechanism by which PDGF regulates selective accumulation of primary, secondary, and cycloheximide-promoted mRNAs is not known. The finding that the PDGF-modulated mRNAs are inhibited by actinomycin D suggests that PDGF may act to selectively stimulate transcription of these mRNAs. Alternatively, PDGF may preferentially increase the translatability of preexisting mRNAs (21). Resolution of this matter requires the use of cloned nucleic acid probes. The present work provides a framework for the exploration of growth factor-modulated gene regulation, a potential regulatory event in the mitogenic response.

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