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Identification of a BALB/c-3T3 Cell Protein Modulated by Platelet-Derived Growth Factor

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The platelet-derived growth factor (PDGF) stimulates density-arrested BALB/c-3T3 cells to synthesize a protein (pII; M_r, 35,000) that is constitutively synthesized by spontaneously transformed BALB/c-3T3 (ST2-3T3) cells which do not require PDGF for growth. Antisera against a major excreted protein family (MEP) of retrovirus-transformed cells quantitatively precipitated cellular pII. PDGF-stimulated pII has the same molecular weight, a similar charge, and similar antigenic determinants as authentic MEP isolated from ST2-3T3 or retrovirus-transformed cells. MEP represented about 2% of the nonnuclear proteins synthesized by ST2-3T3 cells and 0.3 to 0.6% of the proteins synthesized by PDGF-treated BALB/c-3T3 cells, a three- to sixfold increase over the background. In BALB/c-3T3 cells, less PDGF was required for pII (MEP) synthesis than for DNA synthesis. PDGF induced a selective increase in pII (MEP) within 40 min. Such preferential synthesis was inhibited by brief treatment with actinomycin D, suggesting a requirement for newly formed RNA. The constitutive synthesis of pII (MEP) by ST2-3T3 cells was not inhibited by actinomycin D. Five spontaneously or chemical carcinogen-transformed tumorigenic BALB/c-3T3 cell lines were studied; they neither required PDGF for growth nor responded to it. These cell lines became arrested at confluence with a G_1 DNA content. Each of these independently isolated lines synthesized pII (MEP) constitutively. Thus, the synthesis of pII (MEP) may be required, but is not sufficient, for PDGF-modulated DNA synthesis.

Serum contains hormonal polypeptide growth factors which regulate the growth of connective tissue cells in culture (2). One of these proteins, the platelet-derived growth factor (PDGF), is released from the alpha granules of platelets into serum during the clotting process; there is little detectable PDGF in plasma, the liquid portion of unclotted blood (25, 28). PDGF has recently been purified to homogeneity and shown to be a group of highly cationic heat-stable proteins (M_r, 28,000 to 35,000); each member contains two polypeptide chains linked by disulfide bonds (1, 6, 14, 26). Like other polypeptide hormones, homogenous preparations of PDGF are active at 10^{-10} M. Many transformed derivatives of BALB/c-3T3 cells do not require PDGF for growth (27).

PDGF initiates the replication of density-arrested nontransformed BALB/c-3T3 cells by stimulating them to become "competent" to respond to a group of growth factors present in platelet-poor plasma (22). The number of cells rendered competent is a function of both the PDGF concentration and the duration of treatment. A high concentration of PDGF can induce the majority of BALB/c-3T3 cells within a culture to become competent within 0.5 h. Although PDGF binds to tissue culture plates (29), such binding is not responsible for competence; Smith and Stiles (30) have shown that PDGF-treated cells remain competent after trypsinization and transfer to a fresh plate. The subsequent addition of the growth factors to platelet-poor plasma allows these PDGF-treated cells to initiate DNA synthesis after a 12-h lag. The treatment of cells with plasma before the addition of PDGF does not stimulate replication (23). Unlike PDGF, plasma must be present continuously to allow cellular replication (23).

The plasma growth factors have recently been identified (26a, 32). One of these factors is somatomedin (34), a group of proteins with

sequence homology to insulin, whose plasma concentration is regulated by pituitary growth hormone. A high concentration of insulin can replace somatomedin to stimulate growth (32). Epidermal growth factor (EGF; 5) is also needed for an optimal replicative response. PDGF, EGF, or insulin alone induces a weak growth response in density-arrested BALB/c-3T3 cells. In combination, however, these agents function synergistically to stimulate the majority of cells to synthesize DNA (26a).

It has recently been shown that either whole serum (33) or PDGF (21) rapidly stimulates quiescent BALB/c-3T3 cells to preferentially synthesize certain proteins. Such preferential protein synthesis may be required for PDGF-modulated competence because plasma does not stimulate the synthesis of these proteins (21). Furthermore, both the synthesis of these proteins and the acquisition of competence are blocked by inhibitors of RNA synthesis (21, 30). In addition, a spontaneously transformed derivative of BALB/c-3T3 cells (ST2-3T3), which does not require PDGF for growth, constitutively synthesizes several proteins of the same molecular weight as the PDGF-modulated proteins (21). We have studied a group of these transformed cell proteins (pII; M_r, 35,000) in detail because the synthesis of this group increases dramatically in response to PDGF (21). Furthermore, the rate of synthesis of this group approaches that of the actin family. We now show that these proteins share antigenic determinants with major excreted proteins (MEP) secreted by retrovirus-transformed NIH or BALB/c-3T3 cells (10). A group of proteins with similar antigenic determinants, molecular weight, and charge was rapidly synthesized by nontransformed NIH or BALB/c-3T3 cells in response to PDGF.

(A portion of this work has been presented elsewhere in preliminary form [26a].)

MATERIALS AND METHODS

Cell lines. BALB/c-3T3 (clone A31) cells, BP1-3T3 (clone 7-5) cells (a gift of A. B. Pardee), a benzo[*a*]pyrene-transformed BALB/c-3T3 cell line (7, 15, 20), NIH/3T3 cells (clone 1), and a clonal isolate of Kirsten sarcoma virus-transformed nonproducer NIH/3T3 cells (KNIH) (27), free of helper leukemia virus, were grown in Dulbecco modified Eagle medium (DME) containing 10% bovine serum (Colorado Serum Co., Denver, Colo.) on 35-mm tissue culture plates (Lux). ST1-3T3 (clone 8) and ST2-3T3 (clone 2) (21) were independently isolated spontaneous transformants of BALB/c-3T3 cells which were initially recognized because they piled up and overgrew the BALB/c-3T3 cell monolayer. ST2-3T3T is a cell line obtained from a tumor which arose after inoculation of 3×10^6 ST2-3T3 cells into the flank of a nude mouse.

Independently isolated carcinogen-transformed cells were obtained after mutagenesis of BALB/c-3T3 cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1 μg/ml; Sigma Chemical Co., St. Louis, Mo.) or benzo(a)pyrene (10 μg/ml; Sigma). Exponentially replicating BALB/c-3T3 cells were treated with the carcinogen for 2 days or left untreated. The cultures were allowed to grow to confluence in 60-mm plates and replated at 100 cells per plate. This cycle of culture transfer was repeated three times to allow the recognition of independent morphologically transformed colonies, MNNG1-3T3 and BP2-3T3. Both lines were recloned from single cells before being studied.

Purification of pII (MEP) and preparation of antisera. Initial experiments were conducted with rabbit anti-MEP serum prepared by M. Gottesman (National Institutes of Health). To prepare antisera used in later experiments, the protein was purified by a modification of the method of Gottesman and Cabral (11). Briefly, KNIH cells were grown to confluence in 40 150-cm tissue culture plates (Lux). The medium was removed and, after extensive washing with DME lacking serum, replaced with 400 ml of serum-free DME. Twenty-four hours later, the medium was collected, clarified by low-speed centrifugation, dialyzed against water, and lyophilized to dryness. The lyophilized material was taken up in a solution of 1 M NaCl–0.1 M Tris-hydrochloride (pH 8.0) and centrifuged at 100,000 × *g* for 60 min, and the supernatant was concentrated to 1.5 ml with a membrane filter (Pellicon; Millipore Corp., Bedford, Mass.). The concentrated protein was loaded onto a Sephadex (Pharmacia Fine Chemicals, Piscataway, N.J.) G-75 gel filtration column equilibrated with 1 M NaCl–0.1 M Tris-hydrochloride (pH 8.0). The fractions enriched for MEP were dialyzed against 10 mM Tris-hydrochloride (pH 8.0) and applied to a DEAE-52–cellulose ion-exchange column. The proteins were eluted with a linear gradient of NaCl (0 to 0.5 M) in 10 mM Tris-hydrochloride (pH 8.0). The fractions enriched in MEP were pooled, lyophilized to dryness, suspended in 0.5 ml of 1 M Tris-hydrochloride (pH 8.0)–1 M NaCl, and applied again to a G-75 column for final purification. Purification was monitored by applying the fractions to sodium dodecyl sulfate (SDS)-15% polyacrylamide gel electrophoresis followed by staining with Coomassie blue. A New Zealand white rabbit was inoculated subcutaneously with 100 μg of protein at monthly intervals at multiple sites; the initial inoculation was mixed with 4 ml of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.), whereas others were mixed with incomplete adjuvant. The rabbit was bled 2 weeks after the last inoculation. Preimmune serum did not contain detectable antibody to MEP.

Preparation of [^{35}S]methionine-labeled cellular extracts. Experiments were initiated on confluent cultures grown on 35-mm plates (area, 9 cm^2) 3 to 4 days after the last medium change. The cellular density under these conditions (10% serum) is shown (see Table 3). Confluent cultures were transferred to medium containing DME with 2.5% (0.75 mg/liter) of the usual methionine concentration supplemented with 0.3% plasma and growth factors as indicated. At intervals, [^{35}S]methionine (1,000 Ci/mmol; Amersham Corp.) was added to 30 to 50 μCi/ml; 10 to 30 min later,

the cells were rinsed with DME (containing 100% methionine; 30 mg/liter), preparatory to cellular lysis.

Immune precipitation. The cells were scraped into 1 ml of immune precipitation buffer A (11) (0.154 M NaCl, 10 mM Tris-hydrochloride [pH 7.4], 0.05% Nonidet P-40, 0.05% SDS) with a rubber policeman and centrifuged for 5 min in a microfuge (Eppendorf) to remove cellular debris, and the supernatants were saved. The supernatants were spotted onto Whatman 3MM paper and repeatedly washed with 5% trichloroacetic acid (TCA) followed by ethanol to determine the acid-insoluble counts. Equal quantities of acid-insoluble material (usually 2.5×10^5 to 10×10^5 cpm) were diluted to 1 ml in buffer A and incubated with rabbit antiserum to MEP, at antibody excess. After 30 min at 4°C, 50 μl of a 10% suspension of *Staphylococcus aureus* A (immunoglobulin G-sorb; New England Enzyme Center) was added. Thirty minutes later, the immune complex was pelleted in a microfuge (Eppendorf) and washed three times with immune precipitation buffer B (0.154 M NaCl, 0.05 M Tris-hydrochloride [pH 7.4], 2.5 M KCl, 0.5% Nonidet P-40), once with buffer A, and once with distilled water. Appropriate buffers were added for one- or two-dimensional gel electrophoresis.

One-dimensional gel electrophoresis. The immune precipitates or the non-precipitated samples in immune precipitation buffer A were brought up in water containing 62.5 mM Tris-hydrochloride (pH 6.8), 1% SDS, 0.0005% bromophenol blue, 10% 2-mercaptoethanol, and 10% glycerol; they were heated to 100°C for 2 min before application to 10 or 15% polyacrylamide Laemmli (16) slab gels. After electrophoresis, the gels were soaked in 15% TCA for 60 min and processed for fluorography (3). The gels were overlaid with prefogged (17) XAR-5 film (Kodak); the fluorograms were not fully exposed so that the density of the bands approximated the amount of [^{35}S]methionine incorporated. In some experiments, the fluorograms of the gels were scanned with a densitometer (Quick Scan) with an integrating device (Quick Quant; Helena Laboratories, Beaumont, Tex.) to determine the density of the autoradiographic bands. The molecular weight of newly synthesized proteins was estimated from the position of ^{14}C-labeled protein molecular weight standards (Bethesda Research Laboratories).

Two-dimensional gel electrophoresis. Two-dimensional non-equilibrium gel electrophoresis was performed by a modification of the technique of O'Farrell et al. (19). Cells were scraped into electrofocusing sample buffer (9.5 M urea, 2% Nonidet P-40, 5% 2-mercaptoethanol, 1.6% Ampholines [pH 5 to 7; LKB Instruments Inc.], 0.4% Ampholines [pH 3.5 to 10]) and clarified by centrifugation at $1,400 \times g$ for 5 min before application to the pH gradient gels. The immune precipitates were brought up in 100 μl of 10% SDS–10% 2-mercaptoethanol, heated to 100°C for 2 min, and precipitated by the addition of 500 μl of acetone-NH_4OH (5.3:0.3). After collection of the precipitates, the pellets were dissolved in water and reprecipitated twice more with acetone-NH_4OH. They were then suspended in electrofocusing sample buffer for 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. In certain instances, duplicate first-dimension gels were cut into 0.5-cm slices and eluted with water to determine the pH gradient.

Growth factors and metabolic inhibitors. PDGF was purified to homogeneity by a modification of our previously described protocol (1). Because these experiments consumed large quantities of PDGF, many experiments were conducted with PDGF purified through the Bio-Gel P150 stage (the third step) or the carboxymethyl Sephadex stage (the second step) of our protocol (1). Highly purified EGF (5) was purchased from Collaborative Research, Inc., Waltham, Mass., and insulin was purchased from Sigma. Stock solutions of the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Consolidated Midland Corp., Brewster, N.Y.) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2 mg/ml and kept at −20°C. The TPA was diluted into DME and used within 1 h of thawing. Control cultures were treated with DME supplemented with an appropriate concentration of DMSO (0.015%). At this concentration, DMSO had no effect on pII (MEP) synthesis or on cell replication. Human platelet-poor plasma (New England Regional Blood Center, Boston, Mass.) was prepared and dialyzed against 0.15 M NaCl as described elsewhere (22). In some experiments, actinomycin D was used at a concentration of 5 μg/ml; this concentration inhibited greater than 95% of RNA synthesis.

DNA synthesis. Confluent cells were studied 3 days after a medium change. After treatment with PDGF, the density-arrested cells were washed with DME and transferred to medium containing an optimal concentration of plasma (22). For 35-mm plates, on which most of these experiments were performed, this concentration was 30%. The medium was supplemented with [^{3}H]thymidine (5 μCi/ml; 6.7 Ci/mmol). Thirty hours later, 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.

Growth curves. Cultures were taken up with trypsin, and 5×10^4 cells were transferred to 35-mm plates supplemented with DME containing 1% serum. One day later, the medium was removed, and the cells were washed once with serum-free DME and transferred to DME supplemented with either 10% serum or plasma (27). The medium was changed twice weekly. At intervals, duplicate cultures were taken up with lidocaine (24) and counted to determine the mean; the number of cells per culture did not vary by more than 15% in the duplicate cultures. The saturation density was the stable equilibrium density that the cells reached in either serum- or plasma-supplemented medium.

Flow microfluorimetry. The cultures were grown to confluence on 100-mm dishes in 10% serum-supplemented medium. Two days after a medium change, the cultures were taken up with trypsin, concentrated by low-speed centrifugation, and suspended in 3 ml of propidium iodide (0.05 mg/ml in 0.1% sodium citrate). The cells were cooled to 0°C for 15 to 60 min before analysis with a model 4800 A flow microfluorimeter (Biophysics Div. System, Ortho Instruments, Raritan, N.J.) having a 488-M excitation wavelength (32).

Tumorigenicity. Cell cultures were inoculated into four to eight BALB/c athymic nude mice. The mice were maintained under clean but not germfree condi-

tions. Approximately 3×10^6 cells prepared from exponentially replicating cultures were taken up with trypsin and inoculated into the flanks of the mice (32). Cells were classified as tumorigenic when they formed progressively growing tumors of greater than 0.5 cm in diameter in 75% or more of the mice within 4 to 6 weeks of inoculation.

RESULTS

One-dimensional gel electrophoresis. To identify pII, confluent density-arrested ST2-3T3 cells were treated with 0.15 to 1,500 ng of Bio-Gel PDGF per ml or with 0.3 to 30% platelet-poor plasma for 5 h in low-methionine DME before the addition of [^{35}S]methionine. Thirty minutes later, the cultures were harvested and processed for one-dimensional gel electrophoresis with fluorography. A major protein (pII) was noted at 35,000 daltons (Fig. 1). The density of this band (pII) was similar to that of actin. The synthesis of pII was unaffected by the treatment of cells with either PDGF or plasma (Fig. 1), indicating that ST2-3T3 cells produce it constitutively.

Two-dimensional gel electrophoresis. Because the density of pII was similar to that of actin, we could identify it after two-dimensional gel electrophoresis. Confluent ST2-3T3 cells in 30% plasma were labeled with [^{35}S]methionine and processed for two-dimensional gel electrophoresis. Three spots of similar charge were noted at a molecular weight of 35,000. The major spot of this family (presumably pII) was more positively charged than were the others (Fig. 2A). PDGF-treated BALB/c-3T3 cells had two spots at similar positions (Fig. 2B), as did plasma-treated cells (Fig. 2C). The PDGF-treated BALB/c-3T3 cells synthesized larger amounts of this family of proteins than did the plasma-treated cells, but less than did the transformed cells.

Antisera to MEP reaction with pII. Pulse-chase experiments demonstrated that pII was secreted into the medium. The addition of inorganic $^{32}PO_4$ to the cells demonstrated that the secreted protein was phosphorylated. In addition, it appeared to be a glycoprotein because it could be labeled with [^{3}H]glucosamine; furthermore, it became bound to concanavalin A agarose and could be eluted with 0.1 M alpha-methyl-mannoside (data not shown). Gottesman and his collaborators (10–12) have demonstrated that a MEP of KNIH cells and other retrovirus-transformed cells has similar characteristics.

ST2-3T3 cells were studied to learn whether pII shares antigenic determinants with MEP. A confluent culture was treated with plasma-supplemented medium for 2 h before the addition of [^{35}S]methionine; 30 min later, the cells were harvested, and the cytoplasmic extracts were treated with various quantities of antisera to MEP (a gift of M. M. Gottesman) and precipitated with *S. aureus* A. The immune supernatants and precipitates were taken up with SDS electrophoresis buffer and displayed on a one-dimensional gel for fluorography. The antiserum removed pII from the cellular extracts in a concentration-dependent fashion, whereas serum from an unimmunized rabbit (or preimmune serum) did not (Fig. 3). Furthermore, the electrophoresis of the immune precipitates demonstrated the quantitative precipitation of pII (Fig. 3). By determining the percentage of immunoprecipitable counts at antibody excess, it was found that pII represented approximately 2% of the total nonnuclear proteins synthesized. The data demonstrate that pII, a major protein of ST2-3T3 cells, and MEP from KNIH cells share common antigenic determinants.

ST2-3T3 cells were treated with [^{35}S]methionine for various lengths of time; during a 60-min labeling period, the amount of labeled pII (MEP) immunoprecipitated was proportional to the amount of time the cells were treated with [^{35}S]methionine (data not shown). In subsequent experiments, cells were treated with [^{35}S]methionine for 10 to 30 min to study the synthesis of this protein.

PDGF-stimulated pII (MEP) synthesis in density-arrested 3T3 cells. To learn whether PDGF-stimulated pII shares common antigenic determinants with MEP, we stimulated density-arrested BALB/c-3T3 or NIH/3T3 cells with Bio-Gel PDGF or plasma for 4 h, and equal

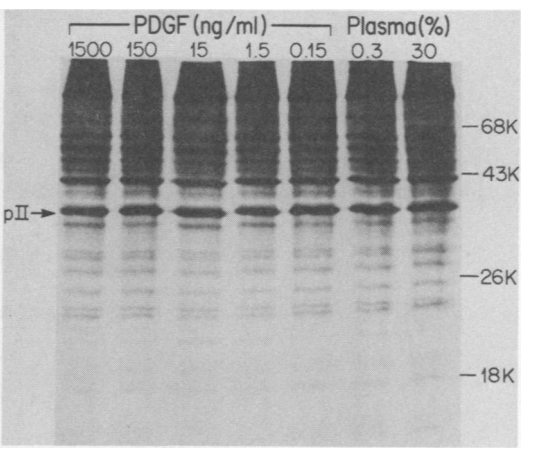

FIG. 1. One-dimensional gel electrophoresis of nonnuclear ST2-3T3 extracts. Confluent ST2-3T3 cells were treated with Bio-Gel PDGF at the concentration indicated in low-methionine DME containing 0.3% plasma or with 0.3 or 30% plasma for 5 h before the addition of [^{35}S]methionine; the cells were harvested 30 min later. Equal quantities of acid-insoluble material (50,000 cpm/sample) were applied to each lane of an SDS-15% polyacrylamide gel. The position of pII and molecular weight markers are shown.

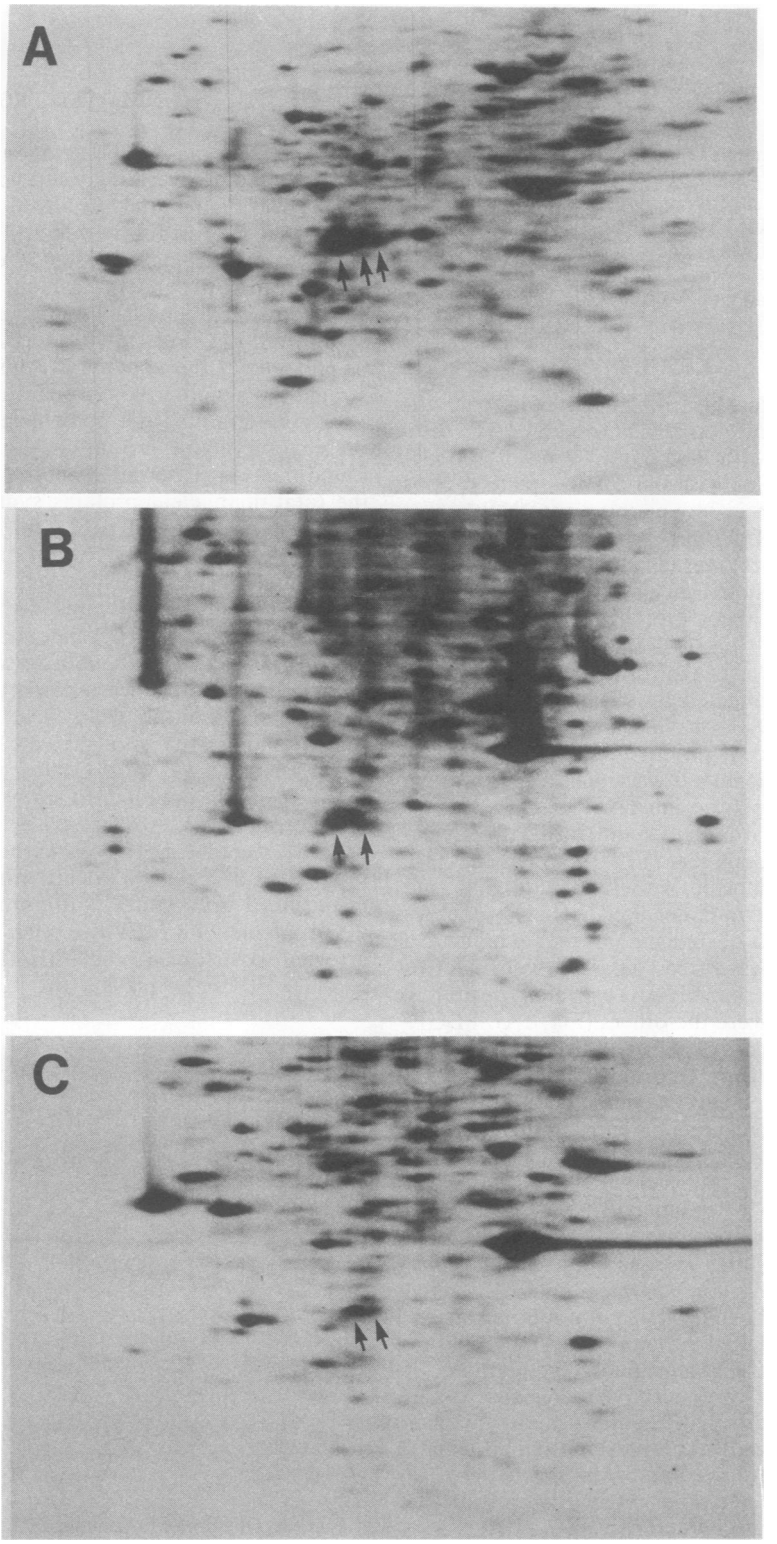

FIG. 2. Two-dimensional gel electrophoresis of nonnuclear extracts: identification of pII. (A) Confluent ST2-3T3 cells were treated with low-methionine DME made up to 30% with platelet-poor plasma for 3 h; [^{35}S]methionine (50 μCi/ml) was then added, and the cells were harvested 30 min later. (B) Density-arrested BALB/c-3T3 cells were treated with carboxymethyl Sephadex PDGF (60 μg/ml) in low-methionine DME containing 0.3% plasma for 4 h before the addition of [^{35}S]methionine; the cells were harvested 30 min later. (C) Density-arrested BALB/c-3T3 cells were treated with 30% plasma for 4 h in low-methionine DME before the addition of [^{35}S]methionine; the cells were harvested 30 min later. For all samples, about 2×10^5 cpm of acid-insoluble material was applied to the gels. The negatively charged pole is to the left, and the positively charged pole is to the right. The arrows indicate pII.

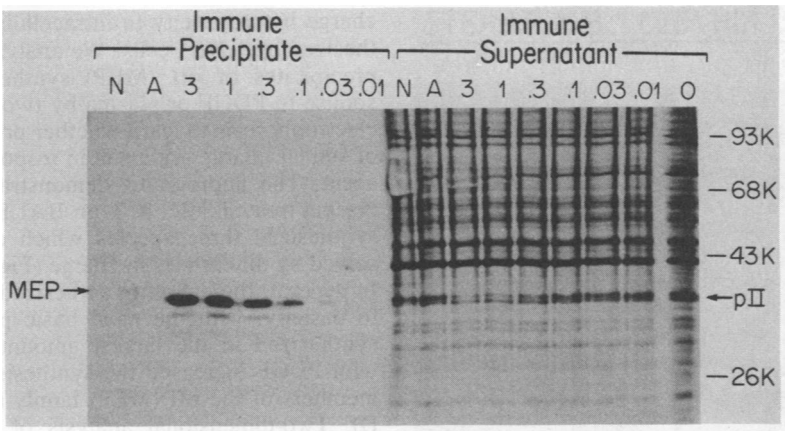

FIG. 3. Quantitative immune precipitation of pII from cellular extracts of ST2-3T3 cells. Confluent ST2-3T3 cells were treated for 2 h with low-methionine DME containing 30% plasma; 50 μCi of [^{35}S]methionine per ml was added, and the cells were harvested for immune precipitation 30 min later. Various quantities (0 to 3 μl as shown) of immune rabbit serum were added to 1 ml of buffer containing equal volumes (50 μl) of cell lysate. The immune complexes were precipitated with *S. aureus* A, and the immune precipitates of supernatants were applied to gel electrophoresis. N refers to 3 μl of normal rabbit serum added to the cell lysate followed by precipitation with *S. aureus* A, whereas A is treatment with *S. aureus* A alone.

quantities of acid-insoluble material were precipitated with an excess of anti-MEP serum for one-dimensional gel electrophoresis. In both cell lines, PDGF stimulated the increased synthesis of an intracellular protein, which shared antigenic determinants with MEP (Fig. 4). The migration of the immunoprecipitated protein from either PDGF- or plasma-treated 3T3 cells was similar to that of authentic intracellular MEP immunoprecipitated from KNIH cells (Fig. 4). It was estimated that pII (MEP) represented approximately 2% of the nonnuclear protein synthesized by the plasma-treated KNIH cells and 0.3 to 0.6% of that synthesized by the PDGF-treated BALB/c-3T3 or NIH/3T3 cells, respectively.

Effect of pure PDGF and other growth-stimulating agents on pII (MEP) synthesis. To demonstrate that the PDGF within this purified preparation rapidly stimulates pII (MEP) synthesis by BALB/c-3T3 cells, we treated confluent cultures with electrophoretically homogenous PDGF (0.4 ng/ml) or plasma for 3 h before labeling. The extracts were immunoprecipitated and analyzed by fluorography after one-dimensional gel electrophoresis. The pII (MEP) band was scanned with a densitometer to quantitate the amount synthesized. The pure PDGF stimulated a 3.7-fold increase in pII (MEP) synthesis compared with plasma-treated cultures (Table 1). As previously shown by Gottesman and Sobel (12), another growth-promoting agent, the tumor promoter TPA, acted similarly to stimulate an increase in pII (MEP) synthesis, in this case, 2.5-fold (Table 1); the concentration used (300 ng/ml) is optimal for DNA synthesis (8). EGF

TABLE 1. Effect of growth-stimulating agents on pII (MEP) synthesis

Agent	Concn	pII (MEP) synthesis[a]	
		BALB/c-3T3	ST2-3T3
Pure PDGF	0.4 ng/ml	370	ND[b]
Bio-gel PDGF	8.1 μg/ml	250	ND[b]
	110 μg/ml	ND[b]	90
TPA	300 ng/ml	250[c]	120[c]
EGF	1 ng/ml	100	ND[b]
	10 ng/ml	140	80
	100 ng/ml	280	160
Insulin	6 μg/ml	120	70
	600 μg/ml	80	ND[b]
Plasma	0.3%	120	100
	30%	100	100

[a] Confluent BALB/c-3T3 or ST2-3T3 cells were treated for 3 h in low-methionine DME containing 0.3% plasma and PDGF, TPA (in 0.015% DMSO), EGF, or insulin. Other cultures were treated with plasma for 3 h with or without 0.015% DMSO; [^{35}S]methionine was added, and the cells were harvested 30 min later. For BALB/c-3T3 and ST2-3T3 cells, 2.5×10^5 and 5×10^5 cpm, respectively were immunoprecipitated and analyzed on one-dimensional SDS gels; the fluorograms were scanned with a densitometer to determine the synthesis of pII (MEP). The density of pII (MEP) is given as the percent plasma control for each cell line and was greater for the plasma-treated ST2-3T3 cells than for the BALB/c-3T3 cells. This table is the composite of several experiments, each of which had a 30% plasma control normalized to 100%.

[b] ND, Not determined.

[c] For the experiments with TPA, the amount of pII (MEP) synthesized in response to TPA is given as the percentage of the control culture treated with plasma and the solvent.

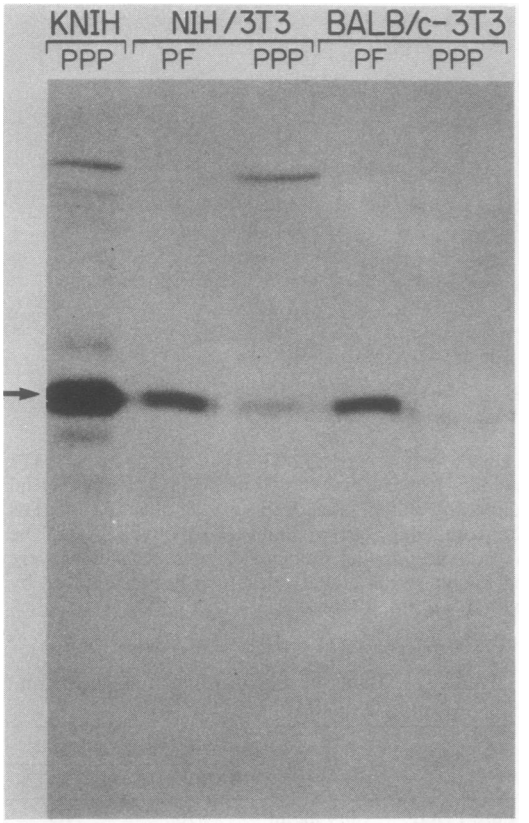

FIG. 4. Increased PDGF-stimulated synthesis of pII (MEP) by nontransformed 3T3 cells. Confluent BALB/c-3T3, NIH/3T3, or KNIH cells were treated for 4 h with 300 μg of Bio-Gel PDGF (PF) per ml in low-methionine DME containing 0.3% plasma or with 30% plasma alone (PPP). [^{35}S]methionine (50 μCi/ml) was added, and 30 min later the cultures were harvested. Equal quantities of TCA-insoluble counts (250,000 cpm/sample) were immunoprecipitated and displayed on a 10% polyacrylamide gel.

stimulated pII (MEP) synthesis at 100 ng/ml, but not at 1 ng/ml; the latter concentration is optimal for DNA synthesis (5). Insulin (6 μg/ml) did not stimulate the selective synthesis of this family of proteins. In contrast to the results with BALB/c-3T3 cells, confluent ST2-3T3 cells showed a less than twofold increase of the synthesis of pII (MEP) in response to PDGF, TPA, or EGF (Table 1). It appears that these transformed cells maximally synthesize pII (MEP) in the absence of these added growth factors. Varying the plasma concentration from 0.3 to 30% had little effect on the selective synthesis of pII (MEP) by either BALB/c-3T3 or ST2-3T3 cells.

Two-dimensional gel electrophoresis of immunoprecipitates from PDGF- or plasma-treated 3T3 cells. Gottesman and Cabral (11) used two-dimensional gel electrophoresis to demonstrate charge heterogeneity in intracellular MEP synthesized by KNIH cells. We analyzed immunoprecipitates of pII (MEP) synthesized in response to PDGF or plasma by two-dimensional electrophoresis to learn whether protein species of similar charge are made in response to either agent. The fluorograms demonstrated that the plasma-treated NIH/3T3 or BALB/c-3T3 cells synthesized three species which could be resolved by differences in charge (Fig. 5A and C). In general, the amount synthesized was related to basicity, with the most basic protein being synthesized in the largest amount. Treatment with PDGF increased the synthesis of all three members of the pII (MEP) family (Fig. 5B and D). Two-dimensional analysis of immunoprecipitates from plasma-treated KNIH cells revealed four clearly defined species (Fig. 6A). Three to four species of protein were also synthesized by the ST2-3T3 cells (Fig. 6B).

Time required for PDGF-modulated pII (MEP) synthesis. Confluent BALB/c-3T3 cells were transferred to low-methionine DME for 1 h before continuous treatment with Bio-Gel PDGF. At intervals, [^{35}S]methionine was added, and 10 min later the cultures were harvested and analyzed for pII (MEP) synthesis. The amount of pII (MEP) synthesized from 20 min to 24 h after the addition of PDGF was compared with that synthesized within the first 10 min of PDGF addition. The addition of PDGF stimulated the increased synthesis of pII (MEP) within 40 min (Fig. 7). The amount synthesized continued to increase until 7 h after PDGF addition. From 7 through 24 h after PDGF addition, the amount of pII (MEP) synthesized was approximately ninefold greater than at time zero.

Concentration dependence of PDGF-modulated pII (MEP) synthesis and DNA synthesis. Duplicate cultures of confluent BALB/c-3T3 cells were treated with various concentrations of Bio-Gel PDGF for 4.5 h to stimulate pII (MEP) synthesis or DNA synthesis. One group of cultures was labeled with [^{35}S]methionine to study pII (MEP) synthesis. Another was transferred to medium lacking PDGF, but containing plasma and [^{3}H]thymidine; the cultures were fixed 30 h later and processed for autoradiography to study DNA synthesis. Less PDGF was required to induce pII (MEP) synthesis than was needed for DNA synthesis (Fig. 8). A PDGF concentration of 150 ng/ml stimulated a 1.8-fold increase in pII (MEP) synthesis compared with plasma-treated controls, but did not stimulate an increase in DNA synthesis. Maximal pII (MEP) synthesis was noted at 1,500 ng of PDGF per ml; this concentration stimulated a 3.5-fold increase over plasma-treated controls, but only stimulated 2% of the cells to synthesize DNA. Increasing the PDGF concentration to 7,500 ng/ml did

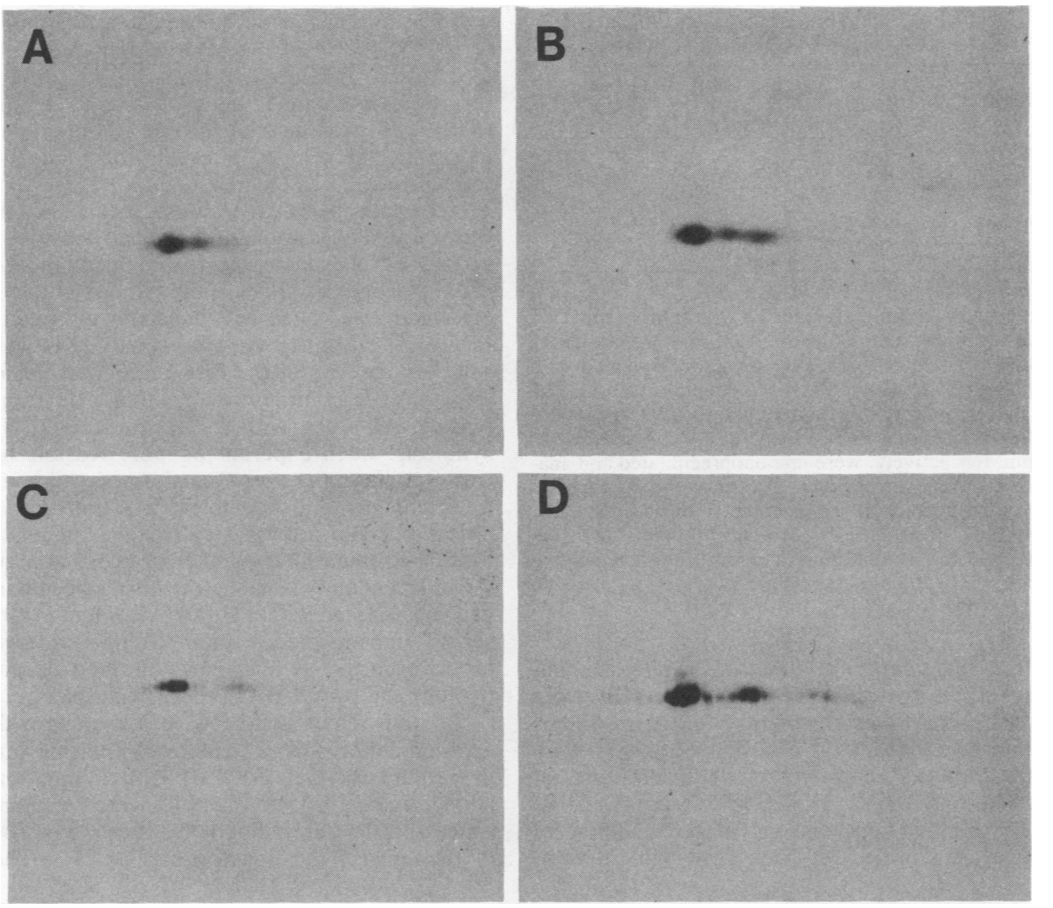

FIG. 5. Two-dimensional gel electrophoresis of immunoprecipitable pII (MEP) from PDGF- or plasma-treated 3T3 cells. Confluent NIH/3T3 cells in low-methionine DME were treated with 30% plasma (A) or 33 μg of Bio-Gel PDGF per ml (B) for 5.5 h. Confluent BALB/c-3T3 cells were treated with 30% plasma (C) or 110 μg of Bio-Gel PDGF per ml (D) for 3 h. In either case, the concentration of PDGF used was in excess over the minimal concentration required for optimal pII (MEP) synthesis. [^{35}S]methionine was added for 30 min before harvest. Equal quantities of TCA-insoluble counts (1×10^6 cpm/sample) were immunoprecipitated and analyzed by fluorography after non-equilibrium pH gradient gel electrophoresis. The negatively charged pole is to the left, and the positively charged pole is to the right. The gels for the NIH/3T3 (A and B) and BALB/c-3T3 (C and D) samples were run separately and had slightly different pH gradients.

not stimulate a further increase in pII (MEP) synthesis, but induced 30% of the cells to synthesize DNA. Thus, pII (MEP) synthesis is more sensitive to PDGF than is DNA synthesis.

Requirement for RNA synthesis. To learn whether RNA synthesis is required for pII (MEP) synthesis, confluent BALB/c-3T3 or ST2-3T3 cells were treated with actinomycin D (5 μg/ml) for 60 min to inhibit >95% of RNA synthesis (data not shown). The cultures were then treated with PDGF or plasma and analyzed for pII (MEP) synthesis 3 h later. The addition of actinomycin D inhibited PDGF-modulated pII (MEP) synthesis by the BALB/c-3T3 cells (Table 2). In contrast, actinomycin D had no effect on the amount of pII (MEP) constitutively synthesized by ST2-3T3 cells. Thus, RNA synthesis appears to be required for PDGF-modulated pII (MEP) synthesis by BALB/c-3T3 cells but, in the short term, not for constitutive synthesis by the transformed cells.

Constitutive synthesis of pII (MEP) by transformed BALB/c-3T3 cell lines. Both ST2-3T3 and KNIH cells synthesize pII (MEP) constitutively and do not require PDGF for replication (27). Thus, the constitutive synthesis of pII (MEP) may be a general property of cells which do not require PDGF for growth. As a test of this hypothesis, five independently isolated morphologically transformed BALB/c-3T3 cell lines were isolated, cloned from single cells, and studied. One (MNNG1-3T3) was transformed

TABLE 2. Inhibition of PDGF-modulated pII (MEP) synthesis by actinomycin D

Agent	Actinomycin D	pII (MEP) synthesis[a]	
		BALB/c-3T3	ST2/3T3
PDGF	Yes	120	80
	No	570	90
Plasma	Yes	120	110
	No	100	100

[a] Confluent BALB/c-3T3 or ST2-3T3 cells were treated (or left untreated) for 1 h with actinomycin D (5 μg/ml) in low-methionine medium in 0.3% plasma as shown. Some cultures were then supplemented with Bio-Gel PDGF (3 μg/ml). [^{35}S]methionine was added 3 h later for a 30-min period before harvesting. For BALB/c-3T3 and ST2-3T3 cells, 1.0×10^5 and 5×10^5 cpm, respectively, were immunoprecipitated and analyzed on one-dimensional SDS gels; the fluorograms were scanned with a densitometer to determine the synthesis of pII (MEP). The density of pII (MEP) is given as the percent plasma control for each cell line incubated without actinomycin D.

after treatment with *N*-methyl-*N'*-nitrosoguanidine, and two (BP1-3T3 and BP2-3T3) were transformed after treatment with benzo[*a*]pyrene. Two lines (ST1-3T3 and ST2-3T3) were spontaneously transformed derivatives of BALB/c-3T3 cells. Unlike the parent BALB/c-3T3 cell line, suspensions of the majority of these transformed cell lines were highly tumorigenic in athymic nude mice (Table 3); tumors formed in ≥75% of the mice within 45 days of inoculation. BALB/c-3T3 cells did not grow to form tumors within 120 days (27). One cell line (ST2-3T3T) was developed by explanting a tumor which developed after the inoculation of transformed ST2-3T3 cells.

Unlike the parent BALB/c-3T3 cell line, the transformed lines grew to similar cell densities in serum- or plasma-supplemented medium. Because plasma lacks PDGF, this experiment demonstrated that these cell lines do not require PDGF for sustained growth (Table 3). To substantiate the loss of the PDGF requirement for growth, it was necessary to treat the transformed cell lines with PDGF to study DNA synthesis. Such experiments were feasible because unlike most virus-transformed cell lines, these chemically or spontaneously transformed clones become growth arrested at confluence. Flow microfluorimetry demonstrated that all of the independently isolated transformed cell lines became arrested with a G_1 DNA content in 10% serum-supplemented medium (Table 3); growth arrest occurred at a higher saturation density than for the parent BALB/c-3T3 cell line.

To study DNA synthesis, confluent growth-arrested cultures were treated with plasma with or without Bio-Gel PDGF. [^{3}H]thymidine was added, and 30 h later the cells were fixed and processed for autoradiography. Less than 10% of the cells of the transformed clones synthe-

TABLE 3. Properties of spontaneously and carcinogen-transformed BALB/c-3T3 cell lines

Cell line	Tumorigenicity in nude mice[a]	Maximum cell density[b] (cells/cm^2 [10^4])		Density serum medium/ density plasma medium	% Confluent cells arrested with G_1 DNA content[c]	[^{3}H]thymidine labeled (PDGF+ plasma/ plasma)[d]	pII (MEP) synthesis[e] (PDGF/plasma)	
		10% Serum	10% Plasma				1.5 μg of PDGF per ml	7.5 μg of PDGF per ml
MNNG1-3T3	Yes	20.0	7.1	2.8	85	1.0	0.8	0.9
BP1-3T3	Yes	42.2	24.4	1.7	92	1.5	0.5	ND[f]
BP2-3T3	Yes	27.8	16.7	1.7	80	1.4	0.9	0.9
ST1-3T3	No	22.2	13.3	1.7	88	1.4	1.3	0.9
ST2-3T3	Yes	14.4	15.6	0.9	82	1.8	1.4	1.4
ST2-3T3T	ND[f]	14.4	18.9	0.8	73	3.0	0.7	0.8
BALB/c-3T3	No	7.1	0.7	10.1	95	38	3.5	3.6

[a] Tumorigenicity was tested by inoculating a suspension of 3×10^6 to 5×10^6 cells subcutaneously into the flanks of four to six athymic nude mice.

[b] The saturation density represents the mean of duplicate cultures in serum- or plasma-supplemented medium.

[c] The percentage of confluent cells with a G_1 DNA content was determined by flow microfluorimetry. Cultures were prepared for flow microfluorimetry 3 days after a medium change.

[d] Growth-arrested confluent cultures (area, 0.3 cm^2) were treated with PDGF (7.5 μg/ml) in 0.3% plasma-supplemented medium or with 0.3% plasma alone for 3 h; the PDGF was removed, and the cultures were transferred to 10% plasma-supplemented medium containing [^{3}H]thymidine (50 μCi/ml). The cultures were fixed and processed for autoradiography 30 h later. Less than 5% of the cells in each culture were stimulated to synthesize DNA by the addition of plasma-supplemented medium.

[e] The synthesis of pII (MEP) was determined as described in Table 1, footnote *a*, after a 5-h treatment of the cultures with Bio-Gel PDGF in 0.3% plasma or 0.3% plasma alone. The treatment of cells with 30% plasma did not stimulate the selective synthesis of pII (MEP).

[f] ND, Not determined.

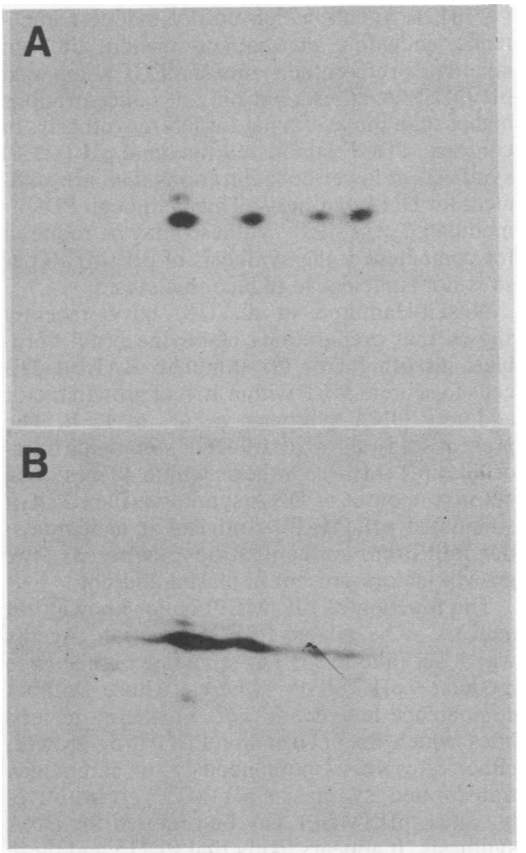

FIG. 6. Two-dimensional gel electrophoresis of immunoprecipitated pII (MEP) from plasma-treated transformed cells. Confluent KNIH (A) or ST2-3T3 (B) cells were treated for 3 h with 30% plasma and labeled for 30 min with [^{35}S]methionine. Equal quantities of TCA-insoluble counts (1×10^6 cpm/sample) were immunoprecipitated and analyzed by fluorography after non-equilibrium pH gradient electrophoresis. The position of the spots can be compared with the position of pII (MEP) synthesized by BALB/c-3T3 cells (Fig. 5C and D), because all of these gels were run at the same time and had similar pH gradients.

sized DNA after the addition of the 5% plasma-supplemented medium; unlike the BALB/c-3T3 cell line, the addition of PDGF (7.5 μg/ml) had little, if any, effect on the percentage of labeled nuclei (Table 3), demonstrating that these transformed cell lines do not synthesize DNA in response to PDGF.

The confluent growth-arrested transformed cell lines were then treated with PDGF to learn whether they respond by selectively synthesizing pII (MEP). The cell lines were treated with Bio-Gel PDGF or with 0.3% plasma for 5 h; the concentrations of PDGF used (1.5 to 7.5 μg/ml) stimulated maximal pII (MEP) synthesis by confluent BALB/c-3T3 cells (Fig. 8). However, un-

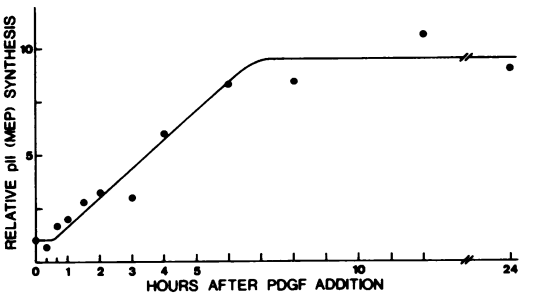

FIG. 7. Increased synthesis of pII (MEP) 40 min after PDGF addition. Density-arrested BALB/c-3T3 cells were transferred to low-methionine DME supplemented with 0.3% plasma. Sixty minutes later, Bio-Gel PDGF (1.6 μg/ml) was added. At the intervals indicated after PDGF addition, the cultures were supplemented with [^{35}S]methionine; they were harvested 10 min later. Equal quantities of acid-insoluble material (150,000 cpm/sample) were immunoprecipitated and analyzed on a one-dimensional polyacrylamide gel. The amount of pII (MEP) synthesized at various times is relative to that synthesized at time zero.

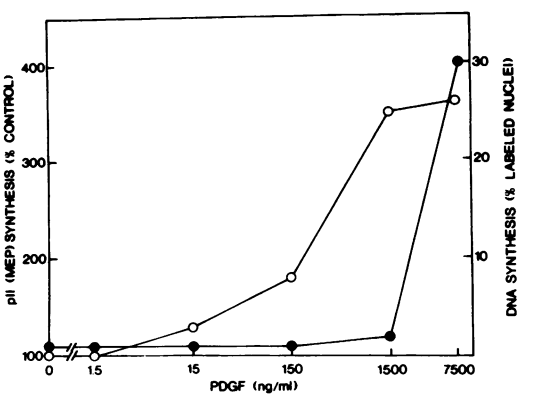

FIG. 8. pII (MEP) synthesis requirement of less PDGF than for competence. Confluent BALB/c-3T3 cells were treated with Bio-Gel PDGF at the concentrations shown in low-methionine DME supplemented with 0.3% plasma for 4.5 h. One group of cultures (○) was labeled with [^{35}S]methionine for 30 min and harvested, and equal quantities of acid-insoluble counts (250,000 cpm/sample) were immunoprecipitated and analyzed on a one-dimensional polyacrylamide gel. The pII (MEP) band was scanned with a densitometer and compared with a 0.3% plasma-treated control culture. A second group of cultures (●) was washed with DME and transferred to a 30% plasma-supplemented medium containing the usual concentration of methionine containing [^{3}H]thymidine (5 μCi/ml). The cells were fixed 30 h later and processed for autoradiography to quantify DNA synthesis. The treatment of BALB/c-3T3 cells in low-methionine medium with PDGF stimulates the cells to become competent to synthesize DNA (32).

like BALB/c-3T3 cells, the transformed cell lines did not increase the synthesis of pII (MEP) in response to PDGF (Table 3). They constitutively synthesized this family of proteins.

DISCUSSION

The present work demonstrates that PDGF rapidly stimulates 3T3 cells to selectively synthesize the pII (MEP) family of proteins. These PDGF-modulated proteins had the same molecular weight and similar charge distribution and antigenicity as a family of proteins constitutively synthesized by transformed, tumorigenic cells which were derived from 3T3 cells. However, the regulation of pII (MEP) synthesis differed in these two cell types.

PDGF-modulated pII (MEP) synthesis in BALB/c-3T3 cells was rapid and began 40 min after PDGF addition. The rate of synthesis continued to increase until 7 h, when it reached a plateau. Two-dimensional gel analysis demonstrated that PDGF stimulates increased synthesis of the same proteins synthesized by plasma-treated cells. PDGF-modulated pII (MEP) synthesis was inhibited by a short treatment with actinomycin D, indicating a requirement for newly formed RNA. In contrast, PDGF did not induce density-arrested transformed cells to synthesize increased amounts of pII (MEP). The constitutive synthesis of this family of proteins by one thoroughly studied spontaneously transformed cell line was insensitive to brief treatment with actinomycin D, indicating the presence of preformed message.

PDGF stimulated BALB/c-3T3 cells to become competent to synthesize DNA. Such cells entered the S phase after transfer to plasma-supplemented medium. The present study shows that five independently isolated transformed cell lines, which neither require PDGF for growth nor respond to it, constitutively synthesize pII (MEP). These lines became transformed either in response to carcinogens or spontaneously after prolonged passage in tissue culture. The synthesis of pII (MEP) by these transformed cells was not due to growth because, like the BALB/c-3T3 cells, these transformed cell lines were confluent and density arrested. Rather, the constitutive synthesis of these proteins may reflect the loss of the PDGF requirement.

Other growth-promoting agents were tested to learn whether they stimulate pII (MEP) synthesis. Tumor promoter TPA, which acts, in part, like PDGF to induce competence (8), also stimulated pII (MEP) synthesis in BALB/c-3T3 cells, but not in transformed cells. TPA acts at a pretranslational level to modulate MEP synthesis in 3T3 cells (12). It also induces MEP synthesis in mouse epidermal cells in vitro (4) and in vivo (13). Agents which do not induce competence, including plasma and insulin, did not stimulate preferential synthesis. EGF stimulated pII (MEP) synthesis but only at concentrations higher than those optimal for DNA synthesis. In contrast, PDGF stimulated maximal pII (MEP) synthesis at lower concentrations than are sufficient for DNA synthesis. Thus, although PDGF-modulated pII (MEP) synthesis may be required for competence, the synthesis of pII (MEP) per se is not sufficient to induce this state.

Nilsen-Hamilton et al. (18) have recently shown that preparations of bovine brain fibroblast growth factor (9) stimulate BALB/c-3T3 cells to secrete MEP within 16 h of growth factor addition; DNA synthesis occurs at 12 h. The present study shows that PDGF stimulates intracellular pII (MEP) synthesis within 40 min, long before the onset of DNA synthesis. Pure PDGF stimulated pII (MEP) synthesis at nanograms-per-milliliter concentrations, whereas the growth factors present in plasma did not.

The function of pII (MEP) is not known, but appears to be related to PDGF action. Agents which act (like PDGF) to stimulate competence modulate pII (MEP), whereas those without competence activity do not. Furthermore, cell lines which do not require PDGF for growth, either retrovirus, spontaneously, or carcinogen transformed, synthesize pII (MEP) constitutively. Thus, pII (MEP) may be required for DNA synthesis. It appears likely that PDGF and these transforming agents induce DNA synthesis, at least in part, by a common mechanism as reflected in the synthesis of this family of proteins.

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