Regulation of the Transcript for a Lysosomal Protein: Evidence for a Gene Program Modified by Platelet-Derived Growth Factor

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Platelet-derived growth factor (PDGF) stimulates density-arrested BALB/c-3T3 cells to synthesize MEP, a lysosomal protein. This enhanced synthesis appears to be largely regulated by the PDGF-modulated accumulation of MEP mRNA, a 1.8-kilobase species. The increase in the MEP transcript, which is dependent on the PDGF concentration, begins 3 to 4 h after PDGF addition and is maximal at 12 h. The accumulation of the MEP transcript is growth-factor specific: PDGF and the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate, an agent which acts like PDGF, induce MEP RNA accumulation, whereas epidermal growth factor, somatomedin C, insulin, and whole plasma do not. A spontaneously transformed BALB/c-3T3 cell line (ST2-3T3), which does not require PDGF for growth, optimally expresses MEP RNA in the absence of PDGF. The PDGF-modulated increase in MEP RNA is unlike PDGF-modulated *c-myc* and *c-fos* RNA accumulation because it is blocked by cycloheximide, suggesting a requirement for de novo protein synthesis. It appears that PDGF modulates a program of gene expression with the accumulation of some transcripts, typified by MEP, being dependent upon the translation of others.

The growth of connective tissue cells in culture, such as the mouse BALB/c-3T3 cell line, is regulated by a group of polypeptide growth factors present in clotted blood serum (5). One of these factors, platelet-derived growth factor (PDGF; M_r 28,000 to 32,000), is a family of glycoproteins composed of two disulfide-linked polypeptide chains (2, 14, 30, 53), one of which is encoded by the *c-sis* protooncogene (16, 35, 65). PDGF appears to initiate the mitogenic response by stimulating cells to become competent to respond to a group of growth factors present in platelet-poor plasma (50), the liquid portion of unclotted blood. The induction of competence has been associated with the increased transcription of certain mRNAs (11, 41), including those encoded by the protooncogenes *c-myc* (37) and *c-fos* (12, 27, 38, 43). Expression of *c-myc* in the absence of PDGF appears to be sufficient to allow a certain percentage of cells to lose their PDGF requirement for growth (3). The accumulation of *c-myc*, *c-fos*, and other PDGF-modulated messages does not require protein synthesis. The addition of cycloheximide does not inhibit the PDGF-modulated accumulation of these protooncogene mRNAs but rather superinduces them (11, 12, 37). Cycloheximide-inhibitable PDGF-modulated mRNAs have been defined only in a cell-free translation system (31) and have not been directly quantified.

PDGF also stimulates the selective synthesis of *c-fos* protein (38, 43) and a family of proteins which do not correspond to known protooncogene products (46, 49). The selective synthesis of the latter proteins is rapid, reaching its peak within a few hours of PDGF addition, long before the onset of DNA synthesis. One of these proteins is a 39,000-dalton phosphorylated glycoprotein termed MEP (24, 25) which carries the mannose-6-phosphate lysosomal recognition marker and is localized to lysosomes (23, 56). Large quantities of MEP are synthesized and secreted by retrovi-

rus-transformed 3T3 cells (24). MEP is also selectively synthesized by nontransformed 3T3 cells after treatment with PDGF (57, 58) or the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (26), two agents which induce competence (21). In addition, carcinogen- or spontaneously transformed BALB/c-3T3 cells, which do not require PDGF for growth, optimally synthesize MEP in the absence of PDGF (57). Thus, the increased synthesis of MEP correlates with the mitogenic response of these cells to PDGF and TPA or to transformation by various agents.

We have recently isolated several recombinant cDNA clones containing MEP sequences and identified a single 1.8-kilobase (kb) MEP RNA transcript in NIH/3T3 cells (15). Absolute levels of MEP mRNA increased after treatment of 3T3 cells with TPA or after transformation by a number of oncogenes, including *v-ras* or *c-ras*. In this work, we studied the accumulation of MEP mRNA in BALB/c-3T3 cells in response to PDGF treatment. We found that PDGF stimulates BALB/c-3T3 cells to accumulate MEP mRNA. We also show that the stimulation of MEP mRNA accumulation is inhibited by cycloheximide, providing evidence that the increase in accumulation of MEP mRNA is dependent upon the translation of preexisting, PDGF-modulated mRNAs.

MATERIALS AND METHODS

Cell lines. BALB/c-3T3 (clone A31) cells and ST2-3T3 (clone 2) cells (49) were used in these experiments. Both cell lines were grown in Dulbecco modified Eagle medium supplemented with 10% bovine serum (Colorado Serum Co., Denver, Colo.). The cultures were grown to confluence on either 100- or 150-mm tissue culture plates (Lux) and used 3 days after a medium change. To determine cell number, cultures were taken up in medium supplemented with 12 mM lidocaine (52) and counted with an electronic cell counter (Coulter Electronics, Hialeah, Fla.).

Growth factors and metabolic inhibitors. PDGF was purified from human platelets (American Red Cross) by a modification of our previously described protocol (2). Because

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these experiments consumed large quantities of PDGF, many experiments were conducted with PDGF that was not as highly purified. These experiments were performed with PDGF purified by heating to 80°C, carboxymethyl cellulose chromatography, and adsorption and elution from Cibron Blue-Sepharose (CL-6B; Pharmacia, Uppsala, Sweden) (30). Highly purified PDGF (about 80% pure) was obtained by passing Blue-Sepharose-purified PDGF through a Bio-Gel P150 column equilibrated with 1 M acetic acid. Purity was estimated by silver staining a preparation analyzed on a sodium dodecyl sulfate (SDS) gel run under nonreducing conditions. PDGF activity during purification was determined on the basis of stimulation of nuclear labeling as described previously (2, 50). In this assay, one unit of PDGF is that quantity which stimulates 10^4 density-arrested BALB/c-3T3 cells to undergo one round of replication. Highly purified epidermal growth factor (EGF) (8) was obtained from Collaborative Research, Inc. (Waltham, Mass.), Biomedical Technologies, Inc. (Cambridge, Mass.), or was a gift of M. Sporn (National Institutes of Health). All three preparations gave similar results. Porcine insulin was obtained from Eli Lilly & Co. (Indianapolis, Ind.), and human somatomedin C (insulin-like growth factor 1) (22, 40, 64) was a gift of R. Furlanetto (Children's Hospital of Philadelphia). Human platelet-poor plasma was prepared from fresh blood drawn at the blood bank of the Children's Hospital of Philadelphia. It was prepared and dialyzed against 0.15 M NaCl as described previously (50). In some experiments cycloheximide (Sigma Chemical Co., St. Louis, Mo.) was used at a concentration of 10 μ g/ml; this concentration inhibited greater than 95% of protein synthesis.

RNA isolation. Total RNA was isolated from the cells by the guanidinium isothiocyanate-cesium chloride method as detailed by Maniatis et al. (42), with the modification that centrifugation was in a SW41 rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 30,000 rpm at 20°C for 24 h. Poly(A)⁺ RNA was isolated as described previously (4).

Northern gel analysis. Poly(A)⁺ RNA was electrophoresed on a 1.5% agarose-formaldehyde submerged horizontal gel (1). Medium endoosmosis agarose (Seakem, Rockland, Maine) was dissolved by heating in E buffer (18 mM sodium dibasic phosphate, 2 mM sodium monobasic phosphate); formaldehyde was added to 6%, and the gel was poured. The running buffer was E buffer containing 3% formaldehyde. After electrophoresis, the RNA was transferred to a nitrocellulose filter (BA85, 0.45- μ m pore size; Schleicher & Schuell, Inc., Keene, N.H.) in 10 $\times$ SSC (1 $\times$ SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) and dried in vacuo for 2 h at 80°C.

Dot-blot analysis. Serial dilutions of whole or poly(A)⁺ RNA in 15 $\times$ SSC were applied to a nitrocellulose filter with a 96-hole Minifold apparatus (Schleicher & Schuell). Alternatively, cytoplasmic contents were treated with formaldehyde as described by White and Bancroft (67). The reaction mixture was heated at 65°C for 10 min, and serial dilutions were dotted onto nitrocellulose filters. All dot-blots were dried in vacuo for 2 h at 80°C before hybridization.

cDNA probes. The isolation and characterization of pMMEP-7, a mouse-derived cDNA clone containing 1.3 kb of cDNA, has been described previously (15). This cDNA was inserted into the *Pst*I site of pBR322; the plasmid containing this insert was grown in *Escherichia coli* HB101. Two mouse ST2-3T3 cell cDNAs (p7C10 and p2A9) inserted into the *Pst*I site of pBR322 were also used. These cDNAs were complementary to mRNAs of 1.8 and 1.0 kb, respectively, that were not regulated by growth factors. These

clones were used as size markers and were also used to show that similar amounts of mRNA were present on the filters.

Nick translation. The p7C10 and p2A9 clones were labeled with [α -³²P]dATP by nick translation (55) at a final DNase I concentration of 50 ng/ml and a DNA polymerase concentration of 200 U/ml. The pMMEP probe was digested with *Pst*I, electrophoresed into agarose, and purified by electroelution before being nick translated. The radioactive probe (in 40 μ l) was precipitated after translation by dilution to 300 μ l with water and addition of 40 μ l of 0.1 M potassium chloride in 100 mM Tris hydrochloride (pH 7.4) followed by addition of 20 μ l of 100 mM spermine (34). The mixture was chilled for 15 min on ice and centrifuged in an Eppendorf microfuge for 10 min at 4°C. The supernatant was removed, and the pellet was suspended in a solution of 75% ethanol in 0.3 M sodium acetate (pH 5.0) and incubated for 1 h on ice. After centrifugation the pellet was dissolved in 50 μ l of 20 mM EDTA (pH 7.0). Before use, the nick-translated probes were denatured by the addition of 0.1 volume of 1 M sodium hydroxide followed by neutralization with 0.33 volume of 0.5 M monobasic sodium phosphate. The nick-translated pMMEP probe was routinely prehybridized for 3 to 4 h at 41°C against pBR322 baked onto nitrocellulose to remove any contaminating pBR322 sequences. Prehybridization was carried out in 2 $\times$ Denhardt solution (1 $\times$ Denhardt solution is 0.02% Ficoll (Pharmacia), 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin) with 50% formamide, 0.1% SDS, 5 $\times$ SSPE (1 $\times$ SSPE is 180 mM sodium chloride, 10 mM monobasic sodium phosphate [pH 7.4], 1 mM EDTA), and 100 μ g of salmon sperm DNA per ml. After prehybridization, the nitrocellulose was removed from the solution containing the probe.

Hybridization. Filters to be probed with pMMEP were prehybridized against a mixture of 5 $\times$ Denhardt solution, 50% formamide, 0.1% SDS, 5 $\times$ SSPE, and 100 μ g of salmon sperm DNA per ml for at least 4 h at 41°C. The pMMEP probe solution was heated at 80°C for 10 min and added to this solution. Hybridization proceeded for 20 h at 41°C. The filters were then washed twice with a solution of 2 $\times$ SSC-0.1% SDS at approximately 25°C and twice more with 0.1 $\times$ SSC-0.1% SDS at 25°C before being blotted dry and fluorographed at -70°C with a Cronex intensifying screen (G. I. du Pont de Nemours & Co., Inc., Wilmington, Del.).

To remove the labeled pMMEP probe, the filters were placed in 1,000 ml of a solution containing 0.1 $\times$ SSPE and 0.1% SDS at 100°C for 2 min. The solution was allowed to cool slowly to room temperature, and the filter was removed. The filter was prehybridized at 65°C for 4 h in a solution containing 10 mM monobasic sodium phosphate, 10 mM dibasic sodium phosphate, 5 $\times$ SSC, 5 $\times$ Denhardt solution, 10 mM EDTA, 0.1% SDS, 30 μ g of poly(A) per ml, and 150 μ g of salmon sperm DNA per ml. The salmon sperm DNA was heated to 100°C for 10 min and then rapidly cooled to 0°C before addition.

The nick-translated p7C10 and p2A9 probes in the hybridization solution were heated to 100°C for 5 min, cooled to room temperature, and added to the prehybridization solution. Hybridization was allowed to proceed at 65°C for 48 h. The filters were then washed four times in a solution of 0.1 $\times$ SSC-0.1% SDS at 50°C for 45 min. The filter was air dried and applied to a Cronex intensifying screen at -70°C.

RESULTS

MEP mRNA accumulation. To learn if PDGF stimulates the accumulation of MEP mRNA in BALB/c-3T3 cells, we transferred density-arrested cells to medium containing 0.3%

platelet-poor plasma with or without the addition of a preparation of Bio-Gel PDGF estimated to be 80% pure. The cultures were harvested 6 h later, and the cytoplasmic contents were treated with formaldehyde, dotted onto nitrocellulose, and hybridized to a labeled pMMEP probe. MEP RNA was found in both the treated and untreated cultures. However, there was approximately a six- to eightfold increase in MEP RNA accumulation in the PDGF-treated cells as compared with that in the untreated controls (Fig. 1).

The mRNAs of both the PDGF-treated and untreated cell cultures were separated by electrophoresis to learn if PDGF-modulated MEP RNA is a single-sized species. Quiescent BALB/c-3T3 cells were treated with Blue-Sepharose-purified PDGF in 0.3% plasma or with 0.3% plasma alone for 6 h. Northern gel analysis of poly(A)⁺ RNA demonstrated the presence of a single MEP mRNA species of 1.8 kb in both the PDGF-treated and untreated cultures (Fig. 2A). The density of the PDGF-modulated band was determined by densitometry and found to be about 17-fold greater than that of the control. To be sure that equivalent amounts of poly(A)⁺ RNA were applied to each lane of the gel, the pMMEP probe was eluted, and the filter was hybridized to two labeled cDNA clones complementary to mouse mRNAs of 1.8 and 1.0 kb which are not regulated by PDGF. A mixture of these two clones hybridized to two species of mRNA whose levels were constant in both the PDGF-treated and untreated cultures (Fig. 2B). Thus, PDGF-modulated MEP mRNA is a single 1.8-kb transcript similar to that found in untreated cells. PDGF selectively increases the accumulation of MEP mRNA compared with two constitutively produced mRNA species.

Concentration dependence and time course. To determine the effect of PDGF concentration on the accumulation of MEP mRNA, we treated density-arrested BALB/c-3T3 cells with various concentrations of Blue-Sepharose-purified PDGF and analyzed MEP mRNA accumulation by using formaldehyde-treated cytoplasmic extracts. PDGF induced MEP mRNA accumulation in a concentration-dependent fashion (Fig. 3, inset). As little as 1.75 μ g/ml (25 U/ml) stimulated a threefold increase in MEP RNA accumulation (Fig. 3). Addition of 35 μ g/ml (500 U/ml) stimulated maximal MEP RNA accumulation, a 12-fold increase, with half-maximal accumulation occurring after the addition of 7 μ g/ml (100 U/ml). From the half-maximal stimulation point, it was determined that 142 U of PDGF activity will stimulate

FIG. 1. Highly purified PDGF stimulates MEP mRNA accumulation in BALB/c-3T3 cells. Two 100-mm-diameter plates (6×10^6 cells) of density-arrested BALB/c-3T3 cells were transferred to medium containing 0.3% platelet-poor plasma (A) or 0.3% plasma supplemented with 3 μ g of Bio-Gel-purified PDGF per ml (specific activity, 200,000 U/mg) (B). Six hours later the cultures were harvested, the cytoplasmic contents were treated with formaldehyde, and serial dilutions were dotted onto nitrocellulose. The cyto-dots were probed with nick-translated pMMEP.

FIG. 2. Northern analysis of PDGF-modulated MEP mRNA. Twenty-two 100-mm diameter plates, containing about 7×10^7 density-arrested BALB/c-3T3 cells, were treated for 6 h with either 0.3% plasma (PPP) or 0.3% plasma with 10 μ g of Blue-Sepharose-purified PDGF (PDGF) per ml (specific activity, 48,000 U/mg). Equal quantities of poly(A)⁺ RNA (14.6 μ g per lane) were electrophoresed on a 1.5% agarose-formaldehyde gel. (A) The RNA was transferred to nitrocellulose and probed with ³²P-labeled nick-translated pMMEP. (B) The pMMEP probe was eluted, and the nitrocellulose filter was re-probed with two nick-translated probes, p7C10 and p2A9, containing cDNAs representative of mRNAs that are not regulated by PDGF.

10^6 cells to display a sixfold increase in MEP mRNA accumulation in 6 h. Similar amounts of PDGF activity induce DNA synthesis in 10^6 quiescent BALB/c-3T3 cells (2).

Density-arrested cultures were treated with PDGF and harvested at intervals to determine the kinetics of MEP mRNA accumulation. An increase in MEP mRNA accumulation was noted 3 to 4 h after PDGF addition (Fig. 4). The amount of MEP mRNA continued to increase for at least 12 h when its level was approximately 12- to 16-fold greater than that found in untreated cells. By 24 h the level of MEP mRNA had decreased somewhat, but was still three- to fourfold greater than that of the control. The synthesis of MEP protein, which was studied in duplicate cultures, for the most part paralleled the accumulation of MEP mRNA (data not shown).

Growth-factor specificity. To learn if the selective accumulation of MEP RNA is specific for PDGF, we treated density-arrested BALB/c-3T3 cells with other growth factors including EGF, insulin, somatomedin C (insulin-like growth factor 1), and platelet-poor plasma. The concentrations of these growth factors covered ranges which are optimal for BALB/c-3T3 cell DNA synthesis (40, 60). These data are summarized in Table 1. Insulin (6 to 600 μ g/ml) did not induce MEP RNA accumulation, nor did EGF (1 to 50 ng/ml). Similarly, somatomedin C (1 to 10 ng/ml) did not induce MEP RNA accumulation; however, at 100 ng/ml a small effect, approximately a twofold increase, was noted. The group of growth factors found in platelet-poor plasma did not induce MEP RNA accumulation. In some experiments, however, transfer of cultures from spent medium containing 10% serum to fresh medium containing 0.3% plasma resulted in a twofold increase. Raising the plasma

FIG. 3. MEP mRNA accumulation is regulated by PDGF concentration. Two 150-mm-diameter plates of BALB/c-3T3 cells (7×10^6 cells) were left untreated (Q) or were treated for 6 h with 0.3% plasma supplemented with various amounts of Blue-Sepharose-purified PDGF (14,000 U/mg) before being harvested for analysis of cytoplasmic MEP mRNA content. Inset, Cytoplasmic RNA dots probed with pMMEP. Q, Quiescent cells in spent medium; O, no PDGF; A, 0.028 $\mu\text{g/ml}$; B, 0.07 $\mu\text{g/ml}$; C, 0.35 $\mu\text{g/ml}$; D, 0.7 $\mu\text{g/ml}$; E, 1.75 $\mu\text{g/ml}$; F, 3.5 $\mu\text{g/ml}$; G, 7.0 $\mu\text{g/ml}$; H, 35 $\mu\text{g/ml}$; I, 70 $\mu\text{g/ml}$; J, 105 $\mu\text{g/ml}$. To determine the relative MEP mRNA accumulation, appropriate nonsaturated cyto-dots were scanned with a densitometer. The values were adjusted by the dilution factor and compared with the intensity of the MEP mRNA dot in quiescent cells which was normalized to 100%.

concentration to 10 or 30% abolished this effect. Addition of Blue-Sepharose-purified PDGF to 0.3% plasma stimulated a 10-fold increase in MEP RNA accumulation. When PDGF was added to 10% plasma, the stimulation in MEP RNA was about half as great. Thus, higher doses of plasma do not stimulate MEP RNA accumulation but in fact inhibit it slightly. This observation is in sharp contrast to the synergism between PDGF and plasma in promoting DNA synthesis (50) and provides evidence that progression through the G_0 - G_1 phase is not required for optimal MEP mRNA accumulation.

PDGF-independent MEP mRNA accumulation. ST2-3T3 cells are a spontaneously transformed tumorigenic derivative of BALB/c-3T3 cells which do not require PDGF for

growth (49, 58). Density-arrested ST2-3T3 cells had approximately fourfold-higher levels of MEP mRNA than quiescent BALB/c-3T3 cells (data not shown). To learn if PDGF stimulates accumulation of MEP mRNA in ST2-3T3 cells, we prepared cytoplasmic RNA from either density-arrested ST2-3T3 cell cultures or cultures treated for 6 h with 0.3% plasma with or without Blue-Sepharose-purified PDGF. The PDGF did not stimulate an increase in MEP RNA accumulation compared with cultures that remained in the spent medium or were treated with plasma alone (Fig. 5). Similarly, PDGF did not induce MEP protein synthesis in a separate group of ST2-3T3 cultures (data not shown) as previously reported (57, 58). The accumulation of MEP protein and RNA is independent of PDGF in density-arrested ST2-3T3 cells.

We have recently shown that the amount of PDGF per cell, rather than the PDGF concentration, regulates the synthesis of both MEP protein and DNA by BALB/c-3T3 cells (59). In the present study, ST2-3T3 cells were treated with a far higher concentration of PDGF than that required for BALB/c-3T3 cells to show optimal expression of MEP RNA, because the former cell line grows to a higher saturation density. The addition of 2,800 U of PDGF per 10^6 ST2-3T3 cells did not stimulate MEP mRNA accumulation. On a per cell basis this amount of PDGF was 20-fold in excess of that necessary to stimulate a half-maximal increase in MEP mRNA accumulation (6-fold) in 10^6 BALB/c-3T3 cells (Fig. 3).

Requirement for protein synthesis. To learn if protein synthesis is required for PDGF-modulated MEP RNA accumulation in BALB/c-3T3 cells, we treated duplicate groups of confluent cultures with Blue-Sepharose-purified PDGF for

FIG. 4. Time course of PDGF-modulated MEP mRNA accumulation. Density-arrested BALB/c-3T3 cell cultures were treated with Blue-Sepharose-purified PDGF (2.7 $\mu\text{g/ml}$; 185,000 U/mg) for the intervals shown. The cultures were harvested, and serial threefold dilutions of cytoplasmic RNA were applied to nitrocellulose and probed with pMMEP.

8 h in the presence or absence of 10 μ g of cycloheximide per ml, a concentration sufficient to inhibit greater than 95% of protein synthesis. PDGF alone induced about a 10- to 30-fold increase in MEP RNA accumulation compared with quiescent cultures. Addition of cycloheximide completely inhibited this increase (Fig. 6A). The pMMEP DNA was then eluted, and the filter was probed with nick-translated p2A9, a clone complementary to an mRNA which is not regulated by PDGF, to demonstrate that similar amounts of mRNA were applied to the dots. Dots prepared from the cultures treated with both PDGF and cycloheximide contained as much p2A9 mRNA as those prepared from the other cultures (Fig. 6B). Thus, cycloheximide specifically inhibited the accumulation of MEP mRNA. Protein synthesis appears to be required for PDGF-modulated MEP RNA accumulation.

DISCUSSION

PDGF stimulates density-arrested BALB/c-3T3 cells to selectively synthesize MEP, a lysosomal protein. Such selective synthesis occurs long before the onset of DNA synthesis and may be required for S-phase entry (57, 58). The present data demonstrate that PDGF regulates MEP synthesis by modulating the accumulation of MEP RNA. PDGF stimulated a 12-fold increase in the level of a single species of MEP RNA in a dose-dependent fashion. This effect was specific for PDGF because other polypeptide growth factors did not induce MEP mRNA accumulation. ST2-3T3 cells, which do not require PDGF for growth, failed to respond to PDGF by accumulating MEP mRNA; rather, they accumulated MEP mRNA in a PDGF-independent fashion. It appears that MEP is a member of a family of gene products that are rapidly and specifically synthesized in response to PDGF. Unlike other PDGF-modulated gene products, however, the accumulation of MEP mRNA requires protein synthesis.

PDGF stimulates quiescent BALB/c-3T3 cells to express

TABLE 1. Effect of growth factors on MEP RNA accumulation

Agent (Concn)	% MEP RNA accumulation ^a
No treatment	100
Plasma (0.3%)	200
(10%)	100
(30%)	100
EGF (1 ng/ml)	100
(10 ng/ml)	100
(50 ng/ml)	100
Insulin (6 μ g/ml)	100
(60 μ g/ml)	100
(600 μ g/ml)	100
Somatomedin C (1 ng/ml)	100
(10 ng/ml)	100
(100 ng/ml)	200
PDGF (7.8 μ g/ml [400 U/ml])	1,000
PDGF + 10% plasma (7.8 μ g/ml [400 U/ml])	500

^a Growth-arrested BALB/c-3T3 cells were treated with the indicated growth factors in 0.3% plasma (except as noted) for 6 h. Dilutions of formaldehyde-treated cytoplasmic extracts were probed with pMMEP. Stimulation was estimated by visual observation and normalized to the MEP RNA content of untreated cells. These results reflect data from several experiments, each of which had an untreated control normalized to 100%.

FIG. 5. ST2-3T3 cells show no increase in MEP RNA accumulation upon treatment with PDGF. Approximately 7×10^6 density-arrested ST2-3T3 cells on 60-mm-diameter plates were left untreated (A), treated with plasma alone (B), or treated with 260 μ g of Blue-Sepharose-purified PDGF per ml (38,000 U/mg) in 2 ml of medium containing 0.3% plasma (C). Six hours later the cultures were harvested. Serial threefold dilutions of cytoplasmic RNA were probed with pMMEP.

c-fos and *c-myc* RNAs. These RNAs are transcribed in a sequential fashion, with *c-fos* RNA appearing within 5 min of PDGF addition and *c-myc* appearing at 30 to 60 min (27). The possibility that *c-myc* expression is regulated by *c-fos* protein is ruled out by the finding that the accumulation of *c-myc* RNA is not inhibited but is superinduced by the addition of cycloheximide (37). In contrast, the PDGF-stimulated accumulation of MEP RNA was sensitive to cycloheximide. Thus, accumulation of the MEP transcript appears to be dependent upon the synthesis of one or more PDGF-modulated proteins. Such a mechanism provides several potential control points for regulation of the PDGF-induced mitogenic signal.

The present study shows that PDGF does not stimulate MEP mRNA accumulation in ST2-3T3 cells, a spontaneously transformed BALB/c-3T3 cell line which does not require PDGF for growth. Furthermore, quiescent ST2-3T3 cells contain more MEP mRNA than density-arrested BALB/c-3T3 cell cultures. We have previously shown (57) that quiescent ST2-3T3 cells synthesize MEP protein at a rate equivalent to, or greater than, PDGF-treated BALB/c-3T3 cell cultures and at a rate approximately 1 order of magnitude higher than that of quiescent BALB/c-3T3 cells.

The mechanism by which ST-3T3 cells have lost their PDGF requirement is of interest. Bowen-Pope et al. (6) have recently shown that cells transformed by a number of agents secrete a PDGF analog. We are presently analyzing ST2-3T3 cells to determine if they synthesize *sis* mRNA and secrete a protein with PDGF-like activity.

It is clear that certain RNAs accumulate in a cell cycle-dependent fashion. The RNAs for p53 (54), Ha- and Ki-ras (7), and a family of mRNAs found in BHK cells (33) all accumulate late in the G₁ phase in serum-treated cells. Another class of mRNAs is induced shortly before or at the time of entry of the cells into the S phase, presumably because the encoded proteins are associated with DNA replication; these proteins include dihydrofolate reductase (32, 39) and histones H1 (51) and H2b (13). At present, it is not clear to what extent the proteins and RNAs expressed by cells leaving G₀ to enter the S phase overlap with those species expressed in exponentially growing cells, which bypass G₀ and enter directly into G₁. Campisi et al. (7) found an increase in the amount of *c-myc* RNA in exponentially growing BALB/c-3T3 cells as compared with that in density-arrested cells. This increase could reflect either growth-

FIG. 6. PDGF-modulated accumulation of MEP mRNA in BALB/c-3T3 cells is dependent upon protein synthesis. Two sets of density-arrested BALB/c-3T3 cells on 100-mm-diameter plates were left untreated (A) or transferred to medium supplemented with 0.3% plasma (B), with 0.3% plasma containing 10 μ g of cycloheximide per ml (C), with 0.3% plasma containing 22 μ g of Blue-Sepharose-purified PDGF per ml (38,000 U/mg) (D), or with 0.3% plasma containing 22 μ g of PDGF per ml and 10 μ g of cycloheximide per ml (E). Eight hours later one set of cells was taken up with lidocaine to determine cell number; the other set was harvested, and the cytoplasmic contents were treated with formaldehyde. The amount of RNA applied to each dot was normalized for cell number, with each undiluted sample containing the RNA from 3.8×10^5 cells. (A) RNA samples probed with nick-translated pMEP. (B) After hybridization, the pMEP probe was eluted, and the dot-blot was hybridized to a cDNA probe (p2A9) complementary to a non-PDGF-modulated mRNA.

factor action or a cell cycle effect. The results of Thompson et al. (62) and Hann et al. (29) which show no alteration in *c-myc* RNA or protein levels during exponential growth of avian cells suggest that *c-myc* transcription may be exclusively a result of growth-factor action. The present study shows that PDGF-induced MEP RNA accumulation in growth-arrested cells is maximal in the presence of 0.3% plasma, a concentration insufficient for optimal stimulation of DNA synthesis by PDGF (50). Addition of plasma to 10%, a level which allows PDGF-treated cells to synthesize DNA (50), causes no further accumulation of MEP RNA. However, accumulation of MEP RNA in exponentially growing cells has not yet been investigated.

We have previously used cell-free translation assays to show that PDGF regulates the level of translatable mRNAs (31). The expression of several of these mRNAs was inhibited by cycloheximide. One of the translation products had an M_r similar, but not identical, to that of the MEP translation product. This PDGF-modulated translation product was not immunoprecipitated by sera directed against the protein backbone of MEP (data not shown). The present study uses nucleic acid hybridization to quantify PDGF-modulated

RNAs and provides direct evidence that MEP RNA accumulation requires protein synthesis.

In addition to PDGF, the tumor promoter TPA also stimulates MEP RNA accumulation (15), resulting in a selective increase in the synthesis of MEP protein (26). Biological studies have shown that PDGF and TPA act, at least in part, in a similar fashion (21). These agents appear to initiate the cell cycle of density-arrested BALB/c-3T3 cells by stimulating them to become competent to synthesize DNA. EGF, somatomedin C (or insulin), and the growth factors in platelet-poor plasma allow PDGF-treated competent cells (but not incompetent cells) to optimally progress through G_0 - G_1 into the S phase (40, 50). The present data demonstrate that these progression factors, at concentrations optimal for DNA synthesis, do not stimulate MEP RNA accumulation. Such accumulation is only induced by factors with competence activity. BALB/c-3T3 cells have previously been shown to display high-affinity receptors for EGF (66) and somatomedin C (10) under the conditions used in the present study. Insulin also binds to density-arrested BALB/c-3T3 cells (61) but appears to stimulate DNA synthesis through its interaction with the somatomedin C receptor (60).

Both agents with competence activity, PDGF and TPA, induce a common gene program, including *c-fos* (27), *c-myc* (37), and MEP mRNAs (15). These agents have a dissimilar structure but appear to act in a similar fashion. A potential link in their action may be the activation of the calcium- and lipid-dependent enzyme protein kinase C. TPA acts directly on this enzyme (9, 45, 47), whereas PDGF may stimulate it by increasing phosphatidylinositol turnover (28) and thus increasing the synthesis of diacylglycerol, a compound which activates protein kinase C.

Although the receptors for EGF (63), insulin (36), and PDGF (17, 20, 44, 48) are tyrosine-specific protein kinases, the differing actions of these agents on MEP RNA indicate that this kinase activity alone cannot account for the growth-factor specificity. Since EGF, insulin, and somatomedin C act in a synergistic fashion with PDGF to promote DNA synthesis, it is not surprising that these progression factors do not modulate the same RNA species as PDGF. EGF appears to have its own distinct effect on a separate class of RNAs (18, 19).

Recently Armelin et al. (3) have shown that the activation of *c-myc* mRNA accumulation in the absence of PDGF stimulates about 30% of density-arrested BALB/c-3T3 cells to synthesize DNA. Thus, there is a coupling between *myc* gene transcription and DNA synthesis, but *myc* transcription alone does not suffice to induce all cells to become PDGF independent. Similarly, dose-response studies have shown that less PDGF is needed to induce MEP protein than is required to make cells competent to synthesize DNA (58). MEP protein synthesis therefore may be required but is not sufficient to allow DNA synthesis. However, MEP is a useful, and as yet a unique, marker of PDGF action because its synthesis is dependent on the translation of the early PDGF-modulated mRNA species. The study of PDGF-modulated gene expression in genetic mutants and hybrid cells should allow a dissection of the PDGF-modulated gene program and define those components essential for cell replication.

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