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Platelet-Derived Growth Factor-Stimulated c-*myc* RNA Accumulation in MG-63 Human Osteosarcoma Cells Is Independent of Both Protein Kinase A and Protein Kinase C

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Treatment of quiescent MG-63 cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or platelet-derived growth factor (PDGF) stimulates the rapid accumulation of c-*myc* RNA. We have now determined that a similar effect can be induced by cAMP. Treatment with forskolin (an activator of adenylate cyclase), IBMX (a phosphodiesterase inhibitor), PGE_1, and isoproterenol stimulated accumulation of both cAMP and c-*myc* RNA, but no increase in either cAMP or c-*myc* RNA was seen with the inactive forskolin analog 1,9-dideoxyforskolin. Forskolin and IBMX acted synergistically in stimulating accumulation of both cAMP and c-*myc* RNA. However, three lines of evidence indicated that PDGF action is not mediated by cAMP. First, PDGF treatment caused no elevation of cAMP within 1 h, even in the presence of IBMX. Second, the kinetics of c-*myc* RNA elevation after treatment with PDGF or forskolin were similar, ruling out delayed onset of cAMP stimulation. Finally, simultaneous treatment with forskolin and the calcium ionophore A23187 enhanced the elevation of c-*myc* RNA levels; no such effect was seen with PDGF. We had previously shown that PDGF action is not affected by prior treatment of MG-63 cells with TPA, a treatment which desensitizes the c-*myc* response to TPA. Similarly, TPA pretreatment had minimal effect on forskolin or IBMX-induced c-*myc* expression. These data suggest that cAMP, phorbol esters, and PDGF act independently to stimulate c-*myc* RNA expression in MG-63 cells. However, nuclear runoff experiments and RNA half-life measurements demonstrated that PDGF, phorbol ester, and cAMP all act to increase the transcription of the *MYC* gene.

The platelet-derived growth factor (PDGF) stimulates c-*myc* gene expression in growth-arrested connective tissue cells (15, 24). Such induction appears to be a critical step in the PDGF-modulated replicative response because c-*myc* expression in the absence of PDGF allows quiescent cells to replicate (1, 21). Thus, the means by which PDGF modulates c-*myc* mRNA levels is of great interest.

Evidence has been presented that PDGF regulates c-*myc* expression in many cell types, in part, by activation of protein kinase C, protein kinase A, or both. The evidence for activation of protein kinase C is compelling. A brief treatment of cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a direct activator of protein kinase C, stimulates c-*myc* RNA accumulation (13). Furthermore, treatment of quiescent 3T3 cells with PDGF increases phosphoinositide metabolism (3, 16), leading to increased production of diacylglycerol; the diacylglycerol, in turn, activates protein kinase C (22). In addition, prolonged pretreatment of 3T3 cells or human fibroblasts with an active phorbol ester, which desensitizes protein kinase C by decreasing the levels of the enzyme (6), causes a 50 to 70% decrease in PDGF-modulated c-*myc* mRNA accumulation (8, 17, 23). Thus, a substantial portion of PDGF-stimulated c-*myc* gene expression in these cells is modulated by protein kinase C, but another portion is not.

In certain cell types, activation of protein kinase A has been implicated in PDGF-modulated c-*myc* expression. PDGF transiently increases the intracellular cyclic AMP (cAMP) concentration of quiescent Swiss 3T3 cells by causing the cells to secrete prostaglandin E_1, which, in turn, activates adenylate cyclase and increases cAMP through a receptor-mediated process (36). Treatment of these cells with forskolin, an activator of adenylate cyclase (38), directly stimulates c-*myc* mRNA accumulation (20, 30), suggesting that cAMP regulates c-*myc* expression. The interpretation of these results is complicated, however, by the fact that forskolin has effects on cells which cannot be attributed to activation of adenylate cyclase (19, 45). Furthermore, stimulation of protein kinase A does not affect all cells in the same way. Induction of cAMP in BALB/c-3T3 cells enhances the ability of PDGF to stimulate DNA synthesis (47), but treatment of growth-arrested BALB/c-3T3 cells with several inducers of cAMP which act by independent mechanisms does not increase c-*myc* mRNA levels (34). Thus, in BALB/c-3T3 cells, PDGF does not stimulate c-*myc* expression by activating protein kinase A.

We have been studying PDGF-modulated c-*myc* RNA accumulation with the ultimate goal of defining the molecular mechanisms by which PDGF stimulates the expression of this gene. Our immediate objective has been to define a cell line in which PDGF stimulates c-*myc* RNA accumulation independently of both protein kinase A and protein kinase C. Such a cell line would be useful because it would allow study of the direct effects of PDGF on c-*myc* expression. The MG-63 human osteosarcoma cell line is potentially useful in this regard because it has already been shown that PDGF stimulates c-*myc* RNA accumulation independently of protein kinase C in these cells (13). Activation of kinase C by phorbol esters stimulated c-*myc* expression in these cells, but desensitization of protein kinase C did not affect PDGF-modulated c-*myc* mRNA accumulation (13). Thus, although

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protein kinase C can induce c-*myc* expression in MG-63 cells, PDGF acts through an independent mechanism.

We have now investigated the role of cAMP in regulating c-*myc* expression in quiescent MG-63 cells. Pharmacologic agents which act by independent mechanisms were found both to increase intracellular cAMP and to stimulate c-*myc* RNA accumulation in growth-arrested cultures. However, PDGF was found to act by an independent means.

MATERIALS AND METHODS

Cells. The properties of MG-63 human osteosarcoma cells (4, 18) have been described (13, 49). A clonal isolate (clone C) (49) was used in these studies. Cultures were grown to confluence on 150-mm-diameter plates in Dulbecco modified Eagle medium (Flow Laboratories, Inc., McLean, Va.) supplemented with 10% (vol/vol) bovine serum (Hyclone Laboratories, Inc., Logan, Utah). They were then transferred to Dulbecco modified Eagle medium containing 0.5% (vol/vol) platelet-poor plasma and used 6 or 7 days later when they had become quiescent (49).

Reagents. Highly purified PDGF (obtained from human platelets) and platelet-poor plasma were prepared as previously described (5, 33). TPA, cholera toxin, prostaglandin E_1 (PGE_1), and 3-isobutyl-1-methyl xanthine (IBMX) were purchased from Sigma Chemical Co., St. Louis, Mo. Forskolin, 1,9-dideoxyforskolin, and L858051 [forskolin, 7-deacetyl-7-(4-methyl-piperazino)-butyryloxydihydrochloride] were purchased from Behring Diagnostics, La Jolla, Calif.). IBMX and PGE_1 were dissolved in ethanol, and forskolin and TPA were dissolved in dimethyl sulfoxide. The final concentrations of ethanol and dimethyl sulfoxide applied to cultures were 0.5 and 0.2%, respectively, and had no effect on c-*myc* expression.

cAMP analysis. Intracellular cAMP levels were analyzed by radioimmunoassay. Growth-arrested cells on 60-mm-diameter dishes (approximately 2×10^6 cells) were treated with the agents to be tested. At various times, the medium was removed, the plates were washed once with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) at 4°C, and the monolayer was inundated with 1 ml of ethanol containing 10 mM HCl. After 10 to 15 min of equilibration, the cell layer was scraped off and the material was vortexed. The extract was clarified by low-speed centrifugation, and the supernatant was collected. The pellet was washed with a 2:1 ratio of ethanol:water; the liquid phases were combined and then evaporated to dryness. The dried material was suspended in acetate buffer supplied by the manufacturer, clarified by low-speed centrifugation, and analyzed for cAMP by radioimmunoassay according to the directions of the manufacturer (Amersham Corp., Arlington Heights, Ill.). Duplicate determinations of the same sample typically varied by less than 10%, whereas separate determinations of duplicate samples varied by 20 to 30%.

RNA analysis. Cytoplasmic RNA was prepared as described by White and Bancroft (48); serial dilutions were baked onto nitrocellulose membranes (Schleicher and Schuell, Inc., Keene, N.H.). For Northern (RNA) blot gel analysis, total cellular RNA was harvested in guanidinium isothiocyanate and pelleted through cesium chloride (7). Equal quantities of RNA were analyzed on 1.5% (wt/vol) agarose gels containing 6% (vol/vol) formaldehyde and transferred to a nitrocellulose membrane (Schleicher and Schuell) by capillary blotting (43). The membranes were hybridized as previously described (13), with the exception that prehybridization and hybridization were performed in 0.5% nonfat dry milk in 5× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate), which had been previously treated with 1% diethyl pyrocarbonate (40). Filters were probed with fragments of recombinant DNA probes which had been purified by electrophoresis through agarose and were labeled by the random primer extension method (12). The large *Pst*I fragment of human c-*myc* 7.4 (46) (a gift of G. Rovera, Wistar Institute, Philadelphia, Pa.) was used. It encompassed 200 base pairs of exon 2, all of exon 3, and 200 base pairs of 3′ noncoding sequences. A cDNA probe of human β_2-microglobulin (42) (a gift of K. Itakura, City of Hope Research Institute, Duarte, Calif.) and plasmid pHA-4.1 (25), a subclone of a genomic human β-actin gene (a gift of R. Weinmann, Wistar Institute), were also used.

In order to quantitate and compare the c-*myc* RNA signal, autoradiograms were scanned with a densitometer (Helena Laboratories, Beaumont, Tex.). Because of the large variation in signal intensity, different exposures of the same autoradiogram were scanned in order to bring all the signals into a measurable range. The autoradiograms shown in the figures were chosen as representatives.

mRNA half-lives. To measure the half-life of c-*myc* RNA, MG-63 cells were stimulated with the appropriate agent for 2 h to achieve peak c-*myc* RNA levels, and then were treated with dactinomycin (5 μg/ml) to halt RNA synthesis. At 15- to 20-min intervals, the cells were washed once with ice-cold PBS and harvested directly in guanidinium isothiocyanate reagent. RNA was subsequently purified through CsCl and analyzed by Northern blotting.

Nuclear run-on. Nuclei were prepared from cells (treated as already described) by the method of Greenberg and Ziff (15). In brief, the monolayer was washed once with PBS, and the cells were scraped into PBS and pelleted at $750 \times g$. The cell pellet was suspended (at 1 ml per plate of cells) in ice-cold nuclear extraction buffer (10 mM Tris hydrochloride [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% [vol/vol] Nonidet P-40) by gentle vortexing and was allowed to incubate for 5 min on ice. The nuclei were pelleted by centrifugation at $750 \times g$ for 5 min at 4°C, washed once with nuclear extraction buffer (2 ml per plate), and repelleted. The washed nuclei were suspended in a solution of 100 μl of 50 mM Tris hydrochloride, pH 8.3, 5 mM $MgCl_2$, 100 μM EDTA, and 40% glycerol, and were frozen at 75°C until use. The nuclei were thawed on ice and added to an equal volume of reaction buffer (10 mM Tris hydrochloride [pH 8.0]; 5 mM $MgCl_2$; 300 mM KCl; 0.5 mM each ATP, GTP, and CTP; 80 μCi of [α-^{32}P]UTP [800 Ci/mmol]). Nascent transcripts were elongated by incubation of the mixture at 30°C for 30 min. The transcription reaction was terminated by addition of guanidium isothiocyanate, and the labeled RNA was purified through CsCl as already described. Prior to hybridization, the RNA was partially hydrolyzed by addition of NaOH to 0.2 M and incubated on ice for 10 min. The alkali was neutralized by the addition of an equal volume of 0.5 M HEPES (free acid) (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) followed by sodium acetate to 0.3 M, and the RNA was ethanol precipitated. Equal counts per minute were used per filter per experiment.

Filters for analysis were prepared by denaturation of intact plasmid DNA which contained the sequences of interest. The plasmids were added to 0.1 M NaOH, heated at 100°C for 15 min, and cooled on ice. Alkali was neutralized by the addition of 1/10 volume of 1 M HCl, and the plasmid preparations were adjusted to 0.25 M Tris hydrochloride [pH 8.0] and 10× SSC by the addition of 1 M Tris hydrochloride [pH 8.0] and 20× SSC. Five micrograms of each plasmid

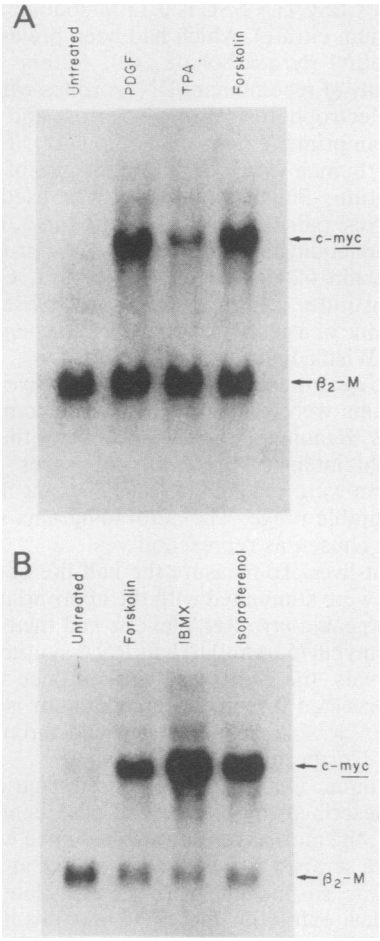

FIG. 1. Agents which regulate cAMP increase c-*myc* mRNA levels. Whole-cell RNA was prepared 2 h after growth-arrested MG-63 cells were treated with (A) PDGF (270 pM), TPA (300 ng/ml), or forskolin (10 μM) or (B) forskolin (10 μM), IBMX (1 mM), or isoproterenol (1 mM), and 25 μg was analyzed via Northern blot transfer. After autoradiography to detect c-*myc* mRNA, the c-*myc* probe was stripped (34) and the filter was rehybridized to a β_2-microglobulin probe. This figure is a composite of the two exposures.

preparation was adsorbed to nitrocellulose with a Bio-Dot SF slot blotter (Bio-Rad Laboratories, Richmond, Calif.), baked, and hybridized to the transcribed RNA as described above.

RESULTS

Induction of c-*myc* RNA. To determine if cAMP can regulate c-*myc* expression, some cultures of quiescent MG-63 cells were either treated with forskolin, an activator of adenylate cyclase (38), or left untreated; as positive controls, other cultures were treated with PDGF or TPA, known inducers of MG-63 cell c-*myc* expression (13). All cultures were harvested 2 h later, and whole-cell RNA was purified and analyzed on Northern blot gels. Forskolin, PDGF, and TPA each stimulated accumulation of the characteristic 2.3-kilobase c-*myc* transcript (Fig. 1A). To be sure that equal amounts of RNA were applied to each lane, the c-*myc* probe was removed by heating and the filter was hybridized to a β_2-microglobulin cDNA probe; β_2-microglobulin mRNA is not regulated by PDGF (13, 24). Similar amounts of β_2-microglobulin mRNA were present in each lane (Fig. 1A).

TABLE 1. PDGF, TPA, and forskolin stimulate c-*myc* expression[a]

Treatment (concn)	Fold increase[b] in mRNA accumulation of:		
	c-*myc* mRNA	β_2-M mRNA	Relative c-*myc* mRNA
None	1.0	1.0	1.0
Forskolin (10 μM)	349	1.0	349
TPA (300 ng/ml)	64	1.3	49
PDGF (270 pM)	335	1.4	239

[a] Growth-arrested MG-63 cells were treated as indicated for 2 h, whole-cell RNA was extracted, and 25 μg of the RNA was analyzed by gel electrophoresis and Northern blot transfer. The RNA response was quantitated by densitometric scanning of the resultant autoradiogram.

[b] The fold increase in c-*myc* and β_2-microglobulin (β_2-M) mRNA accumulation was determined as the ratio of the RNA levels in the treated samples to those in the untreated samples, with the latter values arbitrarily set at 1.0. Relative c-*myc* mRNA was determined as the ratio of the fold increase in c-*myc* RNA accumulation to the increase in β_2-microglobulin RNA for each sample.

In order to compare the levels of c-*myc* RNA after treatment with various agents, the autoradiograms were scanned with a densitometer. Induced levels were compared with the basal c-*myc* RNA level (arbitrarily set at 1.0), and this ratio was taken as the c-*myc* RNA increase (Table 1). The induced level of β_2-microglobulin mRNA in each sample was compared with the untreated control in a similar fashion to account for slight variations in the amount of RNA applied to each lane. By normalization of the c-*myc* level in each sample to the corresponding β_2-microglobulin level, the relative c-*myc* RNA level was obtained. The use of this value corrects for variations in amount of RNA applied per lane and thus permits more precise comparisons of c-*myc* RNA levels in different samples. By this measure, forskolin stimulated a 349-fold increase in c-*myc* mRNA accumulation. The relative increase in PDGF-stimulated c-*myc* RNA was 239, whereas TPA stimulated a 49-fold relative increase (Table 1). Thus, forskolin, an activator of adenylate cyclase, TPA, a modulator of protein kinase C, and PDGF each stimulate c-*myc* expression.

Induction of cAMP. Quiescent cultures were treated with isoproterenol, forskolin, IBMX, or TPA, and the levels of cAMP were determined as a function of time. Forskolin, IBMX, and isoproterenol stimulated a three- to eightfold increase in cAMP within 15 min, whereas TPA was ineffective (Fig. 2A). Forskolin has been reported to have activities dissociable from its ability to elevate cAMP (19, 45). These dissociable activities are also regulated by 1,9-dideoxyforskolin, a forskolin analog which is inactive in stimulating adenylate cyclase (39). To provide additional evidence that activation of adenylate cyclase is necessary for forskolin-induced c-*myc* expression, the ability of forskolin and its analogs to increase intracellular cAMP was determined. Cultures were harvested 15 min after treatment because IBMX-, isoproterenol-, and forskolin-treated cells all have increased levels of cAMP at this time. The water-soluble forskolin analog L858051 (26) was nearly as effective as forskolin was in inducing cAMP, while the inactive congener 1,9-dideoxyforskolin was ineffective (Fig. 2B). The slight increase in the cAMP level caused by 1,9-dideoxyforskolin was within the 20 to 30% variance of the assay. cAMP also increased in response to PGE_1. In summary, forskolin, an activator of adenylate cyclase, IBMX, an inhibitor of phosphodiesterase, and hormones which interact with the β-

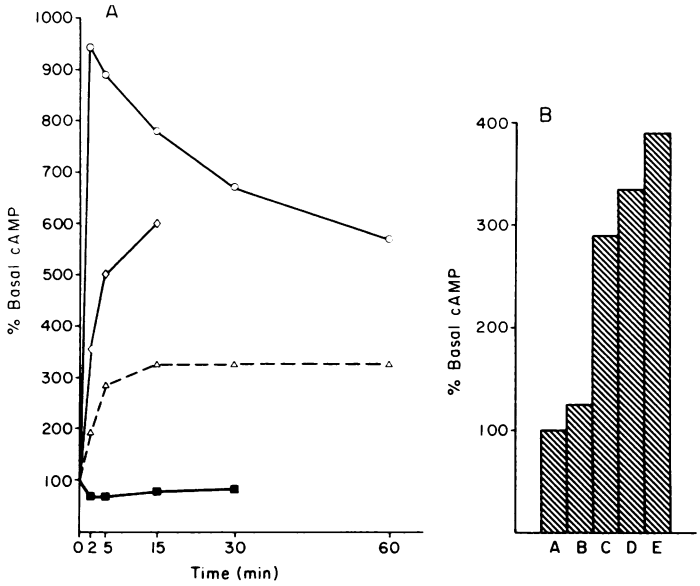

FIG. 2. Stimulation of cAMP levels. (A) Quiescent MG-63 cells were treated with various agents for the times indicated. The cultures were then harvested and extracted for cAMP determination. Treatments and concentrations were as follows: isoproterenol, 1 mM (○); forskolin, 10 μM (◇); IBMX, 1 mM (△); TPA, 300 ng/ml (■). (B) Quiescent MG-63 cells were treated with various agents for 15 min. The cultures were then harvested and extracted for cAMP determination. Cultures were (A) untreated or treated with (B) 10 μM 1,9-dideoxyforskolin, (C) 10 μM L858051, (D) 50 ng of PGE_1 per ml, or (E) 10 μM forskolin.

adrenergic (27) and PGE_1 receptors (37) are all potent inducers of cAMP.

Agents which elevate cAMP stimulate c-*myc* mRNA accumulation. To learn if substances which increase cAMP induce c-*myc* RNA expression, quiescent MG-63 cells were treated with various agents for 2 h, the cytoplasmic RNA was isolated, and the relative c-*myc* RNA levels were determined by using dot blots (Table 2). The agents listed in Table 2 were not compared within the same experiment in a side-by-side fashion. Thus, the data in this table should be considered as qualitative rather than quantitative.

Forskolin and its water-soluble analog, L858051, were found to stimulate a 24- to 25-fold increase in relative c-*myc* mRNA accumulation. In contrast, 1,9-dideoxyforskolin, an analog of forskolin shown in Fig. 2B to be ineffective in increasing cAMP, had no effect on c-*myc* RNA. Isoproterenol, IBMX, and PGE_1, all of which increased intracellular cAMP (Fig. 2), also induced c-*myc* RNA as did cholera toxin, a known activator of adenylate cyclase (14, 41). Thus, agents which increase cAMP—regardless of the mechanism—also stimulate c-*myc* expression. Treatment with dibutyryl cAMP also caused an increase in c-*myc* RNA; in

TABLE 2. Stimulation of c-*myc* RNA by various agents[a]

Treatment	Fold increase in relative c-*myc* RNA
None	1.0
Forskolin (10 μM)	24
L858051 (10 μM)	25
1,9-Dideoxyforskolin (10 μM)	1.3
Cholera toxin (0.5 μg/ml)	62
Isoproterenol (1 mM)	40
PGE_1 (25 ng/ml)	49
IBMX (1 mM)	30

[a] Quiescent MG-63 cells were treated as indicated for 2 h, and the cytoplasmic RNA was extracted and serially diluted for dot blots. The levels of c-*myc* and β_2-microglobulin RNA were quantitated by densitometric scanning of the resultant autoradiograms, and the concentration of c-*myc* RNA was determined relative to that of β_2-microglobulin RNA as described in Table 1, footnote *b*. This table is a compilation of the data from several experiments, each of which had a relative c-*myc* RNA level for the untreated control normalized to 1.0.

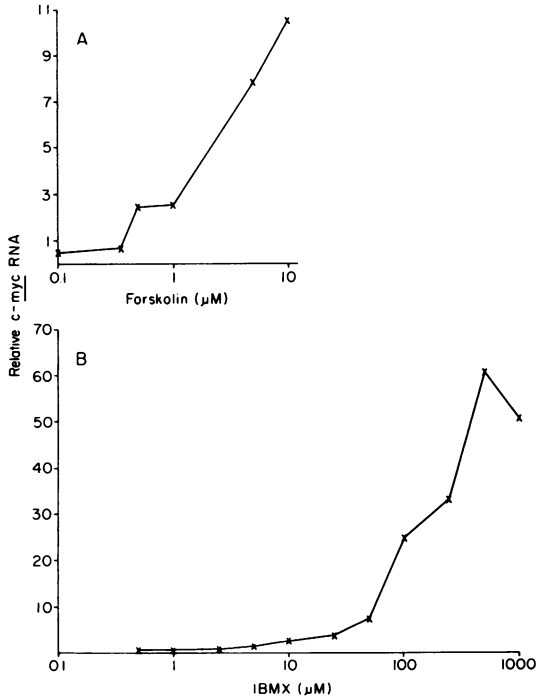

FIG. 3. Forskolin and IBMX stimulate c-*myc* expression in a concentration-dependent fashion. Quiescent MG-63 cultures were treated with various concentrations of either (A) forskolin or (B) IBMX, and the concentration of cytoplasmic c-*myc* RNA was determined relative to that of β_2-microglobulin RNA as described in Table 1, footnote *b*.

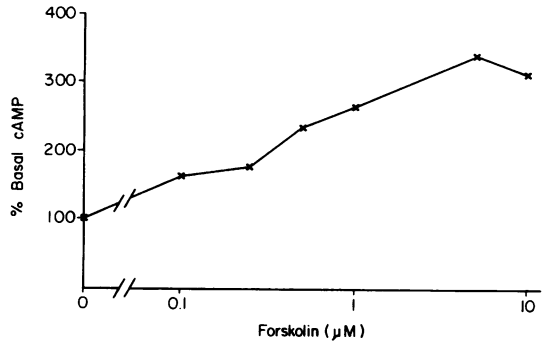

FIG. 4. Forskolin stimulates cAMP in a concentration-dependent fashion. Quiescent MG-63 cells were treated with various concentrations of forskolin. The cultures were harvested 15 min later and analyzed for cAMP by radioimmunoassay. Duplicate samples were obtained, and each was assayed in quadruplicate. The average values are shown.

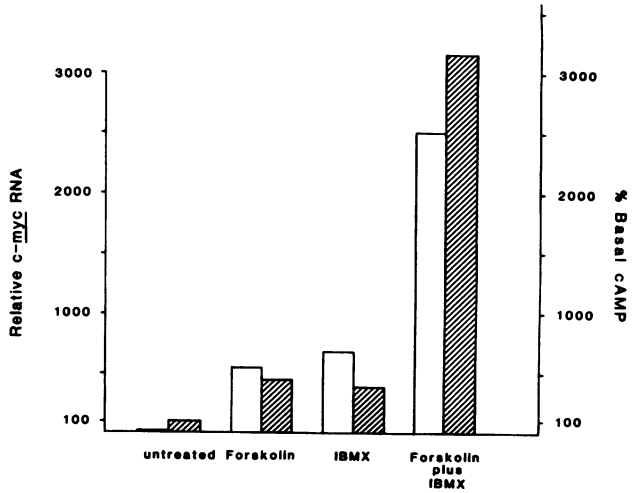

FIG. 5. IBMX acts synergistically with forskolin. Quiescent MG-63 cells were treated with forskolin (10 μM), IBMX (500 μM), or a combination of the two agents or were left untreated. Total RNA was harvested after 2 h, and 50 μg of RNA was analyzed by Northern blot. Parallel cultures were harvested at 15 min and analyzed for cAMP. Symbols: □, c-*myc* RNA; ▨, cAMP.

contrast, neither cGMP nor dibutyryl cGMP caused any discernible increase in c-*myc* RNA levels (data not shown).

To confirm that these agents were all stimulating a similar c-*myc* RNA species, cultures were treated with forskolin, IBMX, or isoproterenol, and total cellular RNA was prepared and analyzed on Northern blot gels (Fig. 1B). Each agent caused accumulation of the same 2.3-kilobase c-*myc* transcript. Clearly, forskolin, IBMX, and isoproterenol stimulate c-*myc* RNA accumulation.

Concentration dependence. The concentration dependence for c-*myc* induction by agents which affect cAMP levels was examined by using cytoplasmic dot blots. Forskolin induced c-*myc* mRNA accumulation as a function of its concentration (Fig. 3A); treatment with 0.5 μM forskolin stimulated a 2.4-fold increase in relative c-*myc* mRNA accumulation, whereas treatment with 10 μM forskolin induced a 10.5-fold increase. Treatment with IBMX also stimulated c-*myc* expression in a concentration-dependent fashion (Fig. 3B), with 500 μM IBMX stimulating a 60-fold increase in relative c-*myc* RNA accumulation.

To demonstrate that the induction of cAMP is also concentration dependent, cultures were treated with various concentrations of forskolin for 15 min and the levels of cAMP were measured. Treatment of cells with 0.1 to 0.25 μM forskolin caused a 60 to 80% increase in cAMP levels, compared with the basal 100% level found in untreated controls. Increasing the forskolin concentration to 5 to 10 μM produced a further increase to 320 to 350% (Fig. 4). There was a correlation between the increase in intracellular cAMP (Fig. 4) and the increase in c-*myc* RNA levels (Fig. 3A).

Synergism of forskolin and IBMX. To further test the hypothesis that the cAMP level controls c-*myc* RNA expression, the ability of a combination of forskolin and IBMX to induce both cAMP and c-*myc* RNA was compared with that of each agent alone. Treatment of cells with either forskolin or IBMX caused an increase in the basal cAMP level from 100 to 400%. Treatment with both agents in combination gave a synergistic response, with a 3,000% increase in the cAMP level (Fig. 5). Such a response was expected because forskolin stimulates adenylate cyclase and IBMX inhibits phosphodiesterase. Similarly, treatment of cells with forskolin or IBMX alone induced a 500- to 700-fold increase in the relative c-*myc* RNA level, as assayed by Northern blot gels. In combination, moreover, there was a 2,500-fold increase in relative c-*myc* RNA accumulation (Fig. 5). Again, the effect of the combination of agents was greater than that of the sum of the individual components demonstrating synergism. These data indicate a clear association between the cAMP level and c-*myc* RNA expression.

PDGF does not modulate intracellular cAMP levels. To determine whether PDGF stimulates c-*myc* RNA levels by increasing cAMP, quiescent MG-63 cultures were treated with PDGF for various lengths of time (up to 60 min) and were harvested to determine the concentration of intracellular cAMP (Fig. 6). PDGF stimulated only a 10% increase. Typically, cAMP levels varied by 20 to 30% between duplicate samples; hence, this 10% increase is within the error of the experiment. To rule out the possibility that PDGF stimulates both adenylate cyclase and phosphodiesterase, causing the destruction of newly synthesized cAMP, cultures were treated with PDGF (or were left untreated) in the presence of the phosphodiesterase inhibitor IBMX. IBMX alone increased the basal cAMP level to 400% within 15 min (Fig. 6). The addition of PDGF had no further effect. Clearly, PDGF does not rapidly increase cAMP in MG-63 cells.

Kinetics of c-*myc* induction. To rule out the possibility of delayed induction of cAMP by PDGF which, in turn, stimulates c-*myc* expression, quiescent cultures were treated with either PDGF or forskolin, and the cells were harvested at various times and assayed for c-*myc* mRNA accumulation by using cytoplasmic dot blots. Both forskolin and PDGF stimulated a 60- to 70-fold relative increase in c-*myc* mRNA, with these maximal levels occurring at 120 to 150 min (Fig. 7). The first increase in response to either agent occurred at 60 min. The decline in the level of c-*myc* mRNA was rapid for each agent and returned to baseline by 4 h. Thus, c-*myc* is induced by forskolin and PDGF with similar kinetics. It does not appear that retarded production of cAMP is responsible for PDGF-modulated c-*myc* RNA accumulation.

Effect of increased Ca^{2+} flux. We have previously shown that treatment of cells with the Ca^{2+} ionophore A23187 in the presence of a normal extracellular concentration of Ca^{2+} (1.8 mM) neither stimulates MG-63 cell c-*myc* expression nor enhances PDGF-modulated expression (13). To determine if increased Ca^{2+} flux affects forskolin-induced c-*myc*

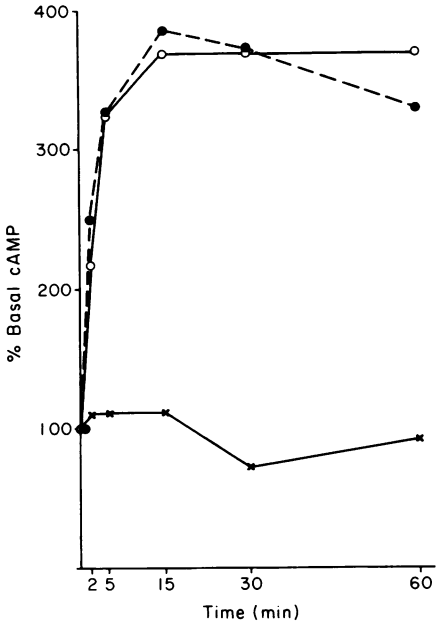

FIG. 6. PDGF does not affect intracellular cAMP levels. Quiescent MG-63 cells were challenged with PDGF (270 pM) (X) alone; in a separate experiment, cultures were treated with IBMX (1 mM) with (●) or without (○) PDGF (270 pM). In both experiments, the cultures were harvested at various times for cAMP analysis. Duplicate samples were obtained, and each was assayed in duplicate. The average values are shown.

expression, quiescent cultures were treated with forskolin alone, A23187 alone, or both agents simultaneously. Northern blot gel analysis revealed that, although A23187 alone was ineffective in inducing c-*myc* mRNA expression, addition to forskolin-treated cells enhanced the forskolin effect (Fig. 8). Quantification of these results demonstrated that A23187 enhanced forskolin-stimulated relative c-*myc* expression approximately 40-fold. The ionophore did not affect the cAMP levels in the forskolin-treated cultures (data not shown). A23187 also enhanced isoproterenol, PGE_1, and cholera toxin induction of c-*myc* mRNA (data not shown),

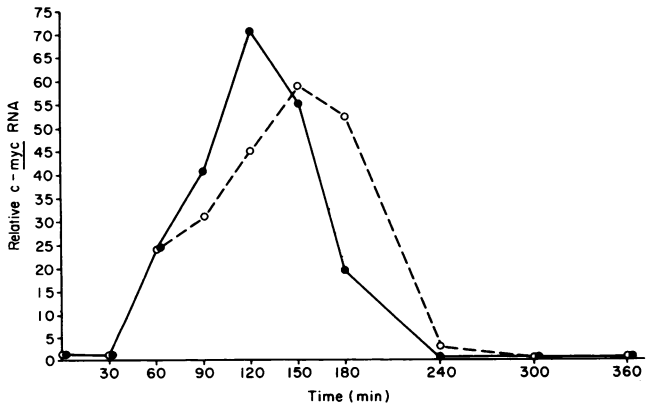

FIG. 7. Kinetics of PDGF- and forskolin-stimulated c-*myc* mRNA accumulation. Quiescent MG-63 cells were treated with PDGF (270 pM) (○) or forskolin (●) (10 μM). The cultures were harvested at various times and analyzed by using cytoplasmic dot blots. The accumulation of c-*myc* RNA is plotted relative to that of β_2-microglobulin RNA.

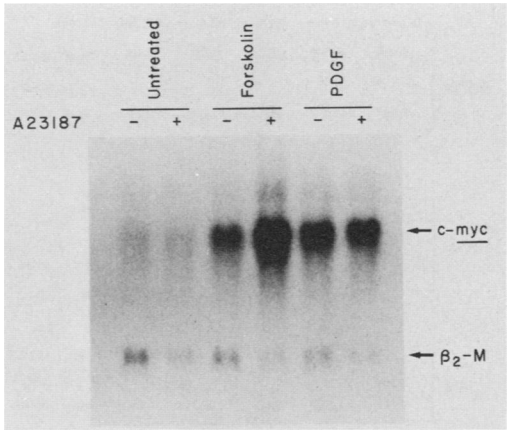

FIG. 8. The calcium ionophore A23187 increases the ability of forskolin to induce c-*myc* RNA accumulation. Quiescent cultures of MG-63 cells were treated (+) or not treated (−) with A23187 (1 μM) as shown. Selected cultures were also left untreated or treated with forskolin (10 μM) or PDGF (270 pM). Total RNA was harvested, and 25 μg of each sample was analyzed on Northern blot gels.

suggesting an effect specific to cAMP-induced c-*myc* expression. In contrast, the ionophore had no effect on PDGF-stimulated c-*myc* RNA accumulation (Fig. 8).

Desensitization of protein kinase C. We have previously shown that PDGF is able to increase c-*myc* RNA levels independently of activation of the phorbol ester receptor, protein kinase C (13). To learn whether cAMP induction of c-*myc* RNA is also independent of protein kinase C, certain cultures were pretreated with TPA for 48 h to desensitize the phorbol ester receptor. Both the TPA-pretreated and nonpretreated cultures were then treated with forskolin, IBMX, or TPA; other cultures were left untreated. RNA was harvested 2 h later, and the c-*myc* and β-microglobulin levels were determined by using Northern blot gels. TPA, forskolin, and IBMX each stimulated c-*myc* expression in the nonpretreated cultures (Fig. 9). Pretreatment inhibited TPA-modulated c-*myc* expression but had little effect on forskolin- or IBMX-modulated expression. These effects were quantified by scanning the gels to determine relative c-*myc* expression. Pretreated cultures accumulated only 3 to 5% of the c-*myc* RNA accumulated in response to TPA by their nonpretreated counterparts. In contrast, pretreated cultures challenged with either forskolin or IBMX accumulated 65 to

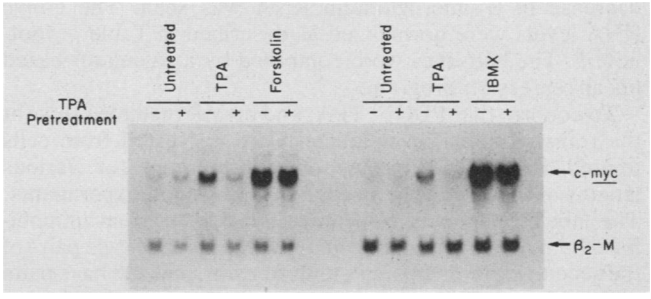

FIG. 9. TPA pretreatment does not effect the ability of forskolin or IBMX to induce c-*myc* RNA. Growth-arrested MG-63 cells were pretreated for 48 h with TPA (100 ng/ml) (+) or left untreated (−) as shown. Certain cultures had no further additions; TPA (300 ng/ml), forskolin (10 μM), or IBMX (1 mM) was added to the other cultures. The cultures were harvested 2 h later, and 50 μg of RNA from each sample was analyzed by Northern blots.

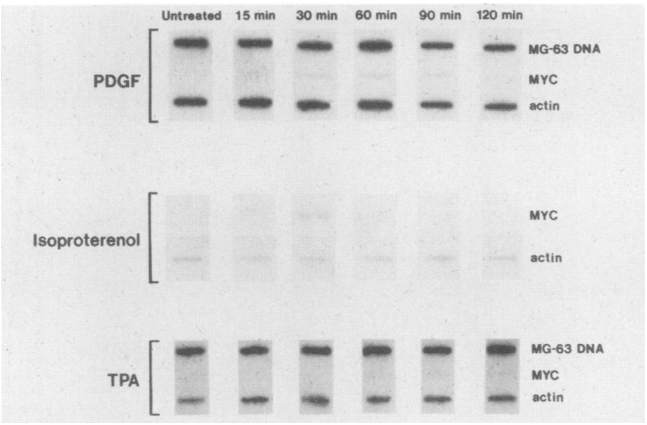

FIG. 10. Quiescent MG-63 cells were treated with PDGF (270 pM), isoproterenol (1 mM), or TPA (300 ng/ml). Nuclei were isolated at the indicated times and used for nuclear runoffs. The RNA products were hybridized against intact plasmids (5 μg each) containing either β-actin or *MYC* (the 3′ end of exon 2, exon 3, and the 3′ untranslated region). MG-63 DNA is an *Eco*RI digest of whole-cell DNA (0.2 μg). The figure shown is a composite of three separate autoradiograms.

72% of the relative c-*myc* RNA accumulated by nonpretreated cultures. Thus, desensitization of protein kinase C does not have a major effect on forskolin- or IBMX-induced c-*myc* expression.

Transcriptional regulation. Expression of c-*myc* RNA accumulation has been shown to be regulated at the transcriptional or posttranscriptional level in various cell systems (11, 29, 32). To learn whether PDGF, cAMP, or TPA increases c-*myc* RNA levels by lengthening the half-life of the message, some cultures of quiescent MG-63 cells were treated with PDGF, TPA, or forskolin for 2 h, whereas others were left untreated. Dactinomycin was then added to inhibit further RNA synthesis, and the amounts of c-*myc* and β_2-microglobulin RNA were quantified by Northern blot gel analysis. The half-life of c-*myc* RNA in the control was 23 min. Neither induction of cAMP (by treatment with forskolin) nor treatment with either PDGF or TPA increased the c-*myc* RNA half-life. In fact, in each of these cases, the c-*myc* RNA half-life was shortened; half-lives were 15, 8, and 13 min when TPA, PDGF, and forskolin were used, respectively. However, simultaneous treatment with PDGF and cycloheximide stabilized the c-*myc* message (data not shown). In the course of the experiment (75 to 100 min), no decrease in β_2-microglobulin RNA was seen. The c-*myc* RNA levels were normalized as described in Table 1, footnote *b*. The half-lives were computed by a calculator-based linear regression program.

To confirm that PDGF, TPA, and cAMP regulate c-*myc* at the transcriptional level, nuclei were harvested from cells treated with PDGF, TPA, or isoproterenol for various lengths of time and were used in nuclear runoff experiments. The labeled nuclear RNAs were hybridized to an immobilized human c-*myc* probe containing the last 200 base pairs of the second exon, the complete third exon, and 200 base pairs of 3′ untranslated region in order to assay completed transcripts. Untreated cells showed no detectable c-*myc* transcription. Treatment with PDGF, isoproterenol, or TPA caused an increase in hybridization to the *MYC* DNA (Fig. 10). A 15-min treatment with isoproterenol or PDGF was sufficient to increase the rate of transcription, with peak transcription occurring at 30 and 60 min, respectively. By 90 to 120 min, the rate of *MYC* transcription in these cultures had returned to baseline. TPA stimulated transcription to a lesser extent consistent with its being a weaker inducer of c-*myc* RNA accumulation (Fig. 1). Forskolin was also capable of inducing *MYC* transcription (data not shown). The transcription rate of β_2-microglobulin was too low to permit its use as a negative control (data not shown). Preliminary experiments revealed that in MG-63 cells, unlike in BALB/c-3T3 cells (15), the transcription of β-actin is not stimulated by PDGF. Thus, in order to determine whether an overall nonspecific increase in transcription was occurring, the nuclear RNA was also annealed to a β-actin probe. The β-actin signal was the same, regardless of the treatment (Fig. 10). Taken together, the RNA half-life and the nuclear run-on experiments show that PDGF, cAMP, and TPA each regulate c-*myc* RNA accumulation at the transcriptional level.

DISCUSSION

The present data provide evidence for the existence of three independent mechanisms which positively regulate c-*myc* expression in quiescent cultures of MG-63 human osteosarcoma cells. First, c-*myc* RNA is expressed in response to activation of protein kinase A by elevations in intracellular cAMP. Second, c-*myc* RNA accumulates when protein kinase C is activated by treatment of cells with a phorbol ester. Third, PDGF regulates c-*myc* RNA levels. In all three of these independent mechanisms, c-*myc* expression is controlled at a transcriptional level.

The evidence that cAMP regulates c-*myc* expression in MG-63 cells is compelling. A variety of agents which act independently to increase intracellular cAMP modulate c-*myc* RNA accumulation. Both the terpene forskolin, which directly activates the stimulatory subunit of adenylate cyclase (38), and IBMX, a known inhibitor of phosphodiesterase, were found to stimulate c-*myc* RNA accumulation in a concentration-dependent fashion. Furthermore, two agents which stimulate adenylate cyclase via a receptor-mediated mechanism, isoproterenol (27), and PGE_1 (37), were also potent inducers of c-*myc* RNA. Cholera toxin, which stimulates adenylate cyclase by inhibiting the inhibitory subunit (14, 41), was also active.

To confirm that these agents stimulate c-*myc* expression by increasing cAMP, intracellular cAMP levels were quantified after treatment; all of the agents which increased cAMP also induced c-*myc* RNA accumulation. Furthermore, forskolin, which was studied in detail, stimulated increases both in intracellular cAMP and c-*myc* RNA in a concentration-dependent fashion. Of note was the finding that 1,9-dideoxyforskolin, a congener of forskolin which does not affect adenylate cyclase, did not increase the concentration of cAMP or c-*myc* RNA. Lastly, treatment of cells with both forskolin and IBMX increased both the cAMP and the c-*myc* RNA concentrations in a synergistic fashion. The c-*myc* RNA level increased approximately 2,500-fold. Thus, the evidence clearly implicates cAMP in the regulation of c-*myc* gene expression.

Because agents which increase cAMP levels are potent inducers of c-*myc* RNA accumulation, it seemed possible that PDGF might stimulate c-*myc* expression in MG-63 cells by modulating cAMP. Precedents for this hypothesis are found in other cell systems. Thyroid-stimulating hormone causes an increase in both the cAMP and c-*myc* RNA concentrations in thyroid epithelial cells (9, 35, 44), as does PDGF in Swiss 3T3 cells (36). The evidence that PDGF

regulates cAMP in Swiss 3T3 cells is clear-cut. In these cells, PDGF stimulates PGE_1 production. PGE_1, in turn, increases intracellular cAMP through a receptor-modulated mechanism (37). As a result, increases in cAMP are delayed compared with those in other hormone systems and are inhibitable by the potent cyclooxygenase inhibitor indomethacin (36). However, evidence that cAMP stimulates c-*myc* in these cells is less compelling. It is based solely on the finding that one agent, forskolin, which has multiple modes of action, stimulates c-*myc* mRNA accumulation (20). It is notable that in BALB/c-3T3 cells, treatment with inducers of cAMP (in the absence of other agents) does not stimulate c-*myc* expression (34).

Although PGE_1 is active in stimulating c-*myc* expression in MG-63 cells, PDGF does not appear to act via a cAMP-dependent pathway. First, PDGF did not increase cAMP levels (within 1 h), even in the presence of the phosphodiesterase inhibitor IBMX. Second, the initial kinetics of PDGF- and cAMP-induced c-*myc* expression were identical. Clearly, a delayed induction of cAMP by PDGF is not responsible for c-*myc* expression. Third, treatment with indomethacin had no effect on the ability of PDGF to induce c-*myc* RNA (data not shown), ruling out cyclooxygenase action and the synthesis of PGE_1 in PDGF-modulated c-*myc* induction. Additional evidence that PDGF and cAMP act independently to stimulate c-*myc* expression is provided by the finding that treatment of MG-63 cells with the calcium ionophore A23187 potentiated the ability of forskolin, but not PDGF, to induce c-*myc* RNA expression.

The present data also show that pretreatment of MG-63 cells with TPA to down regulate protein kinase C does not abolish IBMX- or forskolin-induced c-*myc* RNA expression. Thus, raising intracellular cAMP does not modulate c-*myc* indirectly by activating protein kinase C. Furthermore, TPA does not indirectly increase intracellular cAMP (Fig. 2A).

There are clear-cut differences in the regulation of both c-*myc* expression and cAMP between human MG-63 cells and the mouse 3T3 cell lines, although all are of connective tissue cell origin. First, in BALB/c-3T3 cells (17, 23), as in human fibroblasts (8), a major portion of PDGF-stimulated c-*myc* RNA accumulation is modulated by activation of protein kinase C. In MG-63 cells, PDGF induces c-*myc* independently of protein kinase C (13). Second, in BALB/c-3T3 cells, neither a cAMP agonist (cholera toxin or IBMX) nor A23187 alone is effective in c-*myc* RNA induction; however, the combination of A23187 plus cholera toxin is potent in stimulating c-*myc* RNA accumulation (34). In MG-63 cells, elevation of cAMP alone is sufficient to induce c-*myc* expression, and A23187 potentiates this effect. Third, in Swiss 3T3 cells, PDGF stimulates production of PGE_1 and hence increases cAMP levels; this effect does not occur in MG-63 cells.

The present data, together with those previously reported (13), provide evidence that in MG-63 cells, PDGF, TPA, and cAMP stimulate c-*myc* gene expression by activating independent pathways. However, activation of each of these pathways stimulates *MYC* transcription. No increase in c-*myc* RNA half-life was found after treatment with PDGF, TPA, or inducers of cAMP. Furthermore, nuclear runoff experiments indicate that full-length c-*myc* mRNA is synthesized rapidly after treatment with an agonist. Transcription of *MYC* has been shown to be regulated at two levels, initiation of new transcripts (31) and attenuation (at the exon 1:intron 1 boundary) of previously initiated transcripts (2, 10, 28, 31). Elucidation of the precise mechanisms by which PDGF, TPA, and cAMP regulate *MYC* transcription in MG-63 cells is a high priority in our investigations.

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