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Regulation of 2′,5′-Oligoadenylate Synthetase Gene Expression by Interferons and Platelet-Derived Growth Factor

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In murine BALB/c 3T3 cell cultures, either beta interferon or platelet-derived growth factor (PDGF) enhanced expression of the 2′,5′-oligoadenylate synthetase mRNA and protein. The time course of induction in response to beta interferon was similar to that in response to PDGF. Of several growth factors known to be present in clotted blood serum (i.e., epidermal growth factor, transforming growth factor beta, and PDGF), only PDGF enhanced expression of 2′,5′-oligoadenylate synthetase. The linkage of an interferon response element-containing segment from the 5′-flanking region of a human or murine 2′,5′-oligoadenylate synthetase gene made a heterologous gene responsive to interferon. The expression of such a gene construct in transfected cells was also induced by PDGF. Induction by PDGF was inhibited by mono- or polyclonal antibodies to murine interferon, which suggested that induction by PDGF requires interferon. Both PDGF and interferon induced nuclear factors that bound to this interferon response element-containing segment in vitro.

Many cellular responses to interferons (IFNs) may be mediated by an IFN-regulated biochemical pathway known as a 2′,5′-oligoadenylate system (22, 23, 28, 44). The components of this system include 2′,5′-oligoadenylate synthetases (18, 34), a latent RNase termed RNase L (1, 3, 8, 31), and enzymes degrading 2′,5′-oligoadenylates (including 2′ phosphodiesterases and phosphatases) (17, 39). In the presence of double-stranded RNA, the 2′,5′-oligoadenylate synthetases polymerize ATP into 2′,5′-oligoadenylates which activate RNase L (1, 8, 31). Several forms of 2′,5′-oligoadenylate synthetases have been found in the cell cytoplasm and nucleus (5, 45). Some of these have been isolated in a pure state (11, 47, 49). A human gene encoding two 2′,5′-oligoadenylate synthetases has been cloned and characterized, and several murine genes specifying 2′,5′-oligoadenylate synthetases have been identified (9, 36, 38). Transcription of the human gene encoding two 2′,5′-oligoadenylate synthetases is stimulated by IFN with kinetics which closely parallel the interaction of IFN with cell surface IFN receptors (14). A 2′,5′-oligoadenylate synthetase was shown to inhibit the replication of mengovirus (6). Results of experiments with cell variants differing in RNase L activity make it likely that this inhibitory activity of 2′,5′-oligoadenylate synthetase is mediated by RNase L (21).

The functions of the 2′,5′-oligoadenylate system may not be restricted to antiviral defense (20, 22, 23, 43). Much evidence documents induction of 2′,5′-oligoadenylate synthetase mRNA and protein by mammalian growth factors. The level of 2′,5′-oligoadenylate synthetase was reported to be increased in rat pheochromocytoma cells by nerve growth factor (37) and in quiescent human fibroblasts by epidermal growth factor (EGF) (24). 2′,5′-Oligoadenylate synthetase mRNA and protein were reported to be induced as serum-stimulated mouse embryo fibroblasts traversed the S phase of the cell cycle (48). One of the principal mitogens in mammalian serum, platelet-derived growth factor (PDGF), was found to induce 2′,5′-oligoadenylate synthetase mRNA in quiescent BALB/c 3T3 cells (51).

Here, we describe further observations concerning the induction of 2′,5′-oligoadenylate synthetase mRNA and protein by growth factors and IFN. Our experiments concern (i) the effects of PDGF and IFNs on 2′,5′-oligoadenylate synthetase RNA and enzyme levels, (ii) enhancement by PDGF of the expression of a heterologous gene driven by an IFN response element (IRE)-containing 5′-flanking segment from a 2′,5′-oligoadenylate synthetase gene, (iii) inhibition of this PDGF-promoted enhancement of gene expression by antibodies to IFN and, (iv) induction by PDGF and by IFN of nuclear factors that can bind to the IRE-containing segment from the 2′,5′-oligoadenylate synthetase gene in vitro.

MATERIALS AND METHODS

Abbreviations. Abbreviations used and not previously defined are as follows: bp, base pair; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco modified Eagle medium; kb, kilobase; NIH, National Institutes of Health; OAc, acetate; PBS, phosphate-buffered saline; poly(rI · rC), polyriboinosinic acid · polyribocytidylic acid; TGF-β, transforming growth factor beta.

IFNs and growth factors. Electrophoretically pure mouse IFN-β (specific activity, 10^9 NIH mouse IFN reference units [U] per mg of protein) was produced and purified as previously described (16). Murine IFN doses are given in NIH mouse IFN reference units per milliliter. A homogeneous preparation of recombinant human IFN-α2/α1 was kindly provided by P. Trotta and T. Nagabhushan (Schering Corp., Bloomfield, N.J.). Human IFN doses are given in NIH human IFN reference units per milliliter. A boiled human platelet extract (partially purified PDGF) containing 10,000

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U of PDGF per ml was prepared as described elsewhere (29). Recombinant purified PDGF (B-chain homodimer) was purchased from Amgen, Inc. (Thousand Oaks, Calif.). Recombinant human TGF-β was a generous gift of the Genentech Corp. (South San Francisco, Calif.). EGF was purchased from Collaborative Research, Inc. (Waltham, Mass.) or Sigma Chemical Co. (St. Louis, Mo.). Tumor necrosis factor alpha was generously supplied by M. Shepard (Genentech Corp.). EGF and TGF-β were tested in our laboratory and found to be active (data not shown).

Antibodies. The polyclonal sheep anti-mouse IFN-α/β antiserum and the monoclonal rat anti-mouse IFN-β (7F-D3) antibodies were kindly provided by Y. Kawade and Y. Watanabe (Institute for Virus Research, Kyoto, Japan) (2). A second polyclonal rabbit anti-mouse IFN-α/β antiserum was donated by A. Hovanessian (Pasteur Institute, Paris, France) (13). The polyclonal calf anti-human IFN-β was a gift from J. Vilcek (New York University School of Medicine, New York, N.Y.) (19).

Cells and growth conditions. BALB/c 3T3 (clone A31) cells were grown in DMEM supplemented with 10% calf serum (29). To obtain quiescent monolayers, the cells were split 1:5 on day 0, and the medium was changed on day 3. On day 6, the cells were washed with either DMEM without serum or PBS and grown in DMEM supplemented with human platelet-poor plasma to 5% (vol/vol) for 24 h unless stated otherwise. At this time, the cells were quiescent as documented by [^{3}H]thymidine autoradiography (29). PDGF (300 U/ml) was added unless otherwise stated; this induced DNA synthesis in 95% of the cells (not shown).

Band mobility shift assays. Nuclear extracts were prepared (10, 36) from quiescent BALB/c 3T3 cells that had been treated for 20 min with either 200 U of partially purified PDGF or 1,000 U of recombinant human IFN-α2/α1 per ml (the human IFNα2/α1 is highly active on murine cells [46]); 10,000 cpm of an end-labeled synthetic 29-bp oligodeoxynucleotide containing the IRE from a murine 2',5'-oligoadenylate synthetase gene (which we will designate IRE although it is longer than the minimal IRE; see sequence in the legend to Fig. 6) was incubated with 5 to 10 μg of nuclear extract at 30°C for 30 min according to published procedures (36). A competitor oligodeoxynucleotide (sequence shown in the legend to Fig. 6) was used in binding reactions to confirm the specificity of factor binding to the IRE. After incubation, the reactions were electrophoresed on nondenaturing 4% polyacrylamide gels, using Tris acetate (4) or Tris glycine buffer (40).

Oligodeoxynucleotide probes were synthesized on an Applied Biosystems 380B DNA synthesizer. After synthesis, the oligodeoxynucleotides were deprotected and purified by gel electrophoresis and passage over a Sep-Pak C18 column (Waters Associates, Inc., Milford, Mass.). Single-stranded complementary segments were mixed in equimolar amounts, heated to 65°C for 15 min, and allowed to anneal overnight at room temperature.

Transient transfection assays. DNA (10 μg per plate in 7 ml of medium) was transfected into cells by the calcium-phosphate precipitation method (36). The following modifications were included: 10^6 cells per 100-mm plate were seeded in DMEM with 10% fetal calf serum. At 4 h before DNA transfection, the medium was replaced by DMEM with 2% fetal calf serum. The calcium phosphate-DNA coprecipitate was added and left on the cells for 18 to 20 h. Cell monolayers were then aspirated free of the transfection medium, washed twice with PBS, and incubated in DMEM with 0.5% fetal calf serum for 4 h. At this time the appropriate treatments were carried out (without medium change), and 24 h later the cells were harvested. Harvesting, extraction, and CAT assays were conducted as described elsewhere (36, 42).

Extraction of total cellular RNA. Cells were washed with autoclaved PBS and lysed by addition of 4 M guanidinium isothiocyanate–25 mM NaOAc–100 mM beta-mercaptoethanol (7). The viscous lysates were collected by being scraped with a rubber policeman and gently pipetted into a 15-ml polystyrene tube. The lysates were frozen at −20°C and stored for no longer than 1 week. The frozen lysates were quickly thawed, and the DNA was sheared by vigorous vortexing for 1 min followed by 10 passages through a 20-gauge needle. A 3-ml amount of sheared lysate was gently layered over 2 ml of a 5.7 M CsCl–25 mM sodium citrate cushion and spun in an SW50.1 rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 40,000 rpm and 20°C for 16 h. The RNA pellet fractions were suspended in water, supplemented to 0.3 M NaOAc (pH 5.5), and ethanol EtOH precipitated. The RNA precipitates were resuspended in water and stored at −80°C.

Analysis of RNA products. Total cellular RNA was fractionated by electrophoresis in formamide-formaldehyde agarose gels and transferred to nitrocellulose paper (26). Quantification of total RNA was achieved by UV transillumination of the nitrocellulose filters and direct visualization of ethidium bromide-stained RNA. We find this method more reliable than other internal controls using unique probes. DNA was nick translated by the method of Rigby et al. (33). The cDNA clone (M2) is a 710-bp *Eco*RI fragment encoding the C-terminal portion of a murine 2',5'-oligoadenylate synthetase. This clone was identified in a cDNA library from IFN-treated mouse JLSV-9R cells (38).

Cytoplasmic extracts. Cells in monolayer culture on one 150-mm plate were washed with chilled PBS, scraped with a rubber policeman, and centrifuged at 1,000 × *g* and 4°C for 5 min. The cell pellets were washed in 10 ml of chilled PBS, resuspended in 1 ml of PBS, transferred to 1.5-ml Eppendorf tubes, and centrifuged at 12,000 × *g* and 4°C for 15 s. The cell pellet was suspended in 2 packed-cell volumes of lysis buffer [20 mM *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-KOH (pH 7.4), 10 mM KCl, 1.5 mM $Mg(OAc)_2$, 15 mM beta-mercaptoethanol] supplemented with protease inhibitors as described previously (47). Nonidet P-40 was added to the cell suspension to a concentration of 0.5% (wt/vol), and the mixture was incubated for 10 min with vigorous vortexing every minute. The nuclei were sedimented by centrifugation at 12,000 × *g* and 4°C for 2 min, and the supernatant fraction (cytoplasmic extract) was collected and frozen in equal portions at −70°C.

In-solution 2',5'-oligoadenylate synthetase assay. The in-solution 2',5'-oligoadenylate synthetase assay is a modification of a published method (27). The reaction mixture contained cytoplasmic extract (10 to 20 μg of protein) in 2',5'-oligoadenylate synthetase buffer [20 mM HEPES-KOH (pH 7.4), 120 mM KOAc, 25 mM $Mg(OAc)_2$, 10 mM beta-mercaptoethanol] supplemented with 5 mM ATP, 0.1 μCi of [α-^{32}P]ATP (specific activity, 450 Ci/mmol), and 100 μg of poly(rI · rC). The concentration of poly(rI-rC) was set at 100 μg/ml so that even 2',5'-oligoadenylate synthetase with lower affinity for double-stranded RNA would be detected and quantified (5, 15). The samples were incubated at 30°C for 20 h, and the reactions were terminated by heat inactivation at 95°C for 3 min. The samples were centrifuged at 12,000 × *g* and room temperature for 5 s. The supernatant fraction was incubated with 50 mM $Mg(OAc)_2$, 12 mM

glucose, and 1.25 mg of hexokinase per ml (Sigma) at 30°C for 30 min. Hexokinase converts any remaining ATP to ADP, which can be separated from 2',5'-oligoadenylates in the thin-layer chromatography systems described below (11).

Poly(rI · rC) agarose assay. Cellular extracts were incubated with an equal volume of poly(rI · rC) agarose suspension (Pharmacia, Uppsala, Sweden) in 2',5'-oligoadenylate synthetase buffer at 4°C for 8 h with end-over-end rotation and then centrifuged at 12,000 × *g* and 4°C for 3 min. The pellet fraction was washed once with 150 μl of 2',5'-oligoadenylate synthetase buffer and incubated in 10 μl of this buffer supplemented with ATP and [α-^{32}P]ATP as the in-solution assay. After heat inactivation, the samples were centrifuged at 12,000 × *g* and room temperature for 3 min, and 5 μl of the supernatant fraction was incubated with hexokinase as described above.

Thin-layer chromatography. The reaction mixtures were centrifuged at 12,000 × *g* and room temperature for 25 s, and the supernatant fraction was spotted onto polyethyleneimine-cellulose thin-layer chromatography plates (Sigma). The plates were developed with 0.75 M potassium phosphate (pH 3.5) or 0.25 M ammonium bicarbonate. Both solvent systems were used as a way to check the validity of the results. Products were visualized by autoradiography and quantified with an LKB soft-laser scanner.

RESULTS

Induction of 2',5'-oligoadenylate synthetase mRNAs by partially purified PDGF and IFN-β. In confluent, quiescent, density-arrested BALB/c 3T3 cells, murine IFN-β and partially purified PDGF induced the expression of 2',5'-oligoadenylate synthetase transcripts of similar, if not identical, sizes (Fig. 1A and B). One of these transcripts was about 1.8 kb, and the other was about 4 kb. We reported earlier that IFN induced 2',5'-oligoadenylate synthetase transcripts of both sizes in mouse Ehrlich ascites tumor cells and of the shorter size in mouse JLSV-9R cells (38, 45). The 4-kb transcript could be a precursor of the 1.8-kb transcript; in Ehrlich ascites tumor cells, however, both transcripts were shown to be mRNAs (45).

Both IFN-β and partially purified PDGF induced accumulation of the 4-kb transcript quickly and transiently, with maximal levels (>30-fold and 6-fold, respectively) attained by 3 h in both cases (Fig. 1). In contrast, both IFN-β and partially purified PDGF induced accumulation of the 1.8-kb transcript with slower kinetics (Fig. 1). Maximal induction (15-fold) with PDGF was seen after 9 h. The very high induction ratio of the 4-kb RNA relative to the 1.8-kb RNA, seen with IFN in Fig. 1A, was not always observed. The ratio of 1.8-kb RNA to 4-kb RNA seen after stimulation with PDGF (Fig. 1B) was reproducible.

Induction of a 2',5'-oligoadenylate synthetase by partially purified PDGF. PDGF-mediated induction of 2',5'-oligoadenylate synthetase mRNAs was accompanied by induction of a latent 2',5'-oligoadenylate synthetase activity (Fig. 2). In the experiment shown, quiescent cell cultures were supplemented with 300 U of partially purified PDGF per ml, cell lysates were made 2, 8, 12, and 24 h thereafter, and cytoplasmic 2',5'-oligoadenylate synthetase activity was measured. This activity increased relative to that of controls more than twofold within 2 h and more than eightfold by 8 h. The activity remained elevated for up to 24 h after addition of PDGF (Fig. 2).

Specificity of the response to PDGF. The partially purified PDGF preparations used for the experiments in Fig. 1 and 2

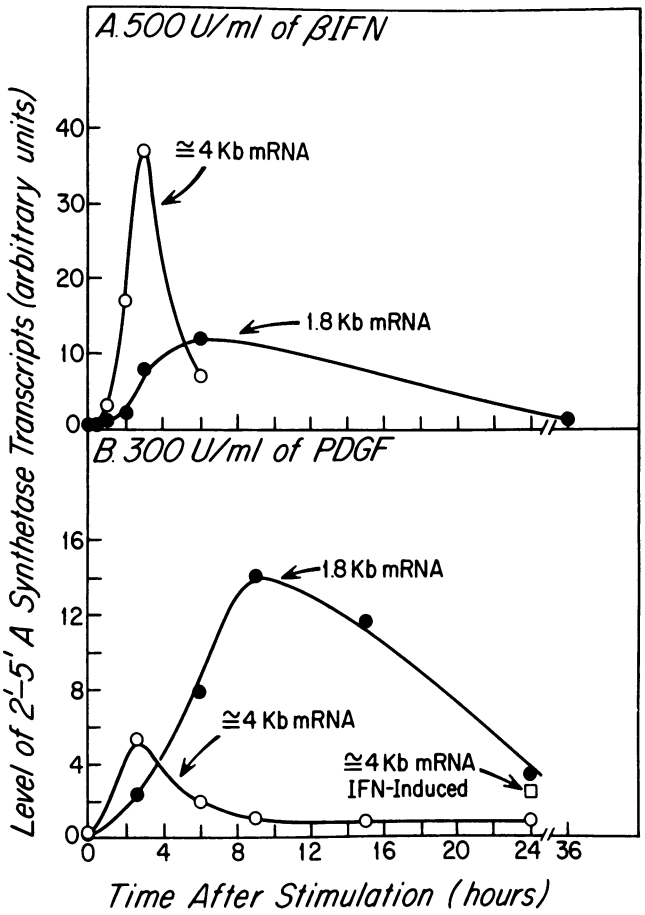

FIG. 1. Time course of induction by IFN-β and partially purified PDGF of expression of 2',5'-oligoadenylate synthetase RNAs in quiescent BALB/c 3T3 cells. Quiescent BALB/c 3T3 cells were treated with 500 U of mouse IFN-β(A) or 300 U of partially purified PDGF (B) per ml for various lengths of time. Total RNA (5 μg) was extracted, purified, fractionated by gel electrophoresis, and transferred to nitrocellulose paper. 2',5'-Oligoadenylate synthetase transcripts were detected by hybridization of the immobilized RNA to a radiolabeled murine 2',5'-oligoadenylate synthetase cDNA probe and autoradiography. The levels of the 1.8- and 4-kb 2',5'-oligoadenylate synthetase transcripts are plotted versus time of exposure to mouse IFN-β or PDGF. Quantification of the autoradiographic signal corresponding to the 2',5'-oligoadenylate synthetase mRNAs was obtained with an LKB soft-laser scanner. The arbitrary units in panels A and B are not the same. For further details, see Materials and Methods.

could have contained other growth factors in addition to PDGF. TGF-β and an EGF-like agent are both contained within platelets and might conceivably have contributed to the responses documented in Fig. 1 and 2. To test whether pure PDGF could induce 2',5'-oligoadenylate synthetase mRNA and to explore the specificity of this response, we treated cells with pure recombinant PDGF alone or in combination with TGF-β or EGF. Electrophoretically pure, recombinant PDGF (B-chain homodimer) induced the expression of a 1.8-kb 2',5'-oligoadenylate synthetase transcript in confluent, quiescent BALB/c 3T3 cells (Fig. 3). (Purified recombinant PDGF also induced accumulation of the 4-kb 2',5'-oligoadenylate synthetase transcript; this is not displayed in Fig. 3 because in the experiment shown, cellular mRNAs were harvested at 9 h after addition of

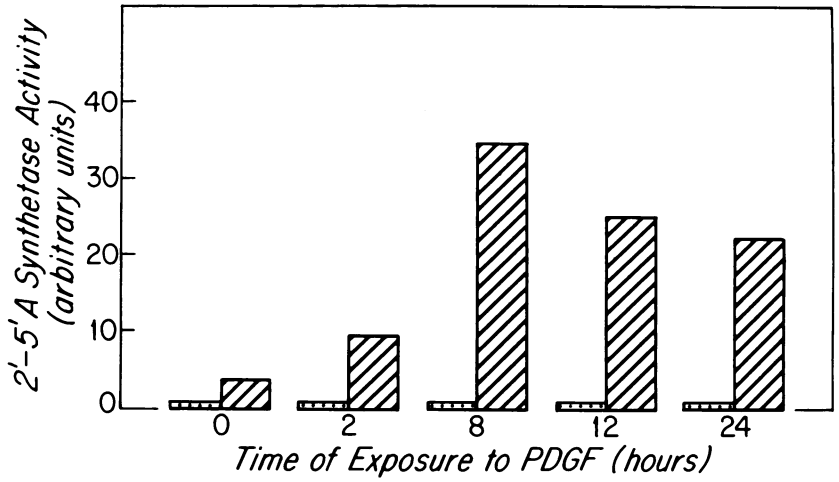

FIG. 2. Time course of induction by partially purified PDGF of expression of 2',5'-oligoadenylate synthetase in quiescent BALB/c 3T3 cells. Quiescent BALB/c 3T3 cells were treated with 300 U of partially purified PDGF per ml for the indicated lengths of time. Cytoplasmic extracts were prepared and assayed for 2',5'-oligoadenylate synthetase activity by the in-solution assay. The relative amounts of 2',5'-oligoadenylate synthetase activity in the absence (▭) or presence (▨) of 100 μg of poly(rI · rC) per ml are shown. These values are plotted versus hours of exposure to PDGF. The values shown represent the averages of duplicate assays which varied by less than 10%. For further details, see Materials and Methods.

PDGF, at which time the 4-kb transcripts were present at only very low levels [Fig. 1].)

Neither the 4-kb nor the 1.8-kb 2',5'-oligoadenylate synthetase mRNA was induced by TGF-β or EGF (each at 20 ng/ml) to any significant degree (Fig. 3). We also determined whether TGF-β or EGF would enhance the response to PDGF. The agonistic effect of these growth factors on induction of the 1.8-kb 2',5'-oligoadenylate synthetase transcript by PDGF was modest (Fig. 3). The concentrations of TGF-β and EGF used in these experiments were in the range at which these factors elicit strong biological responses.

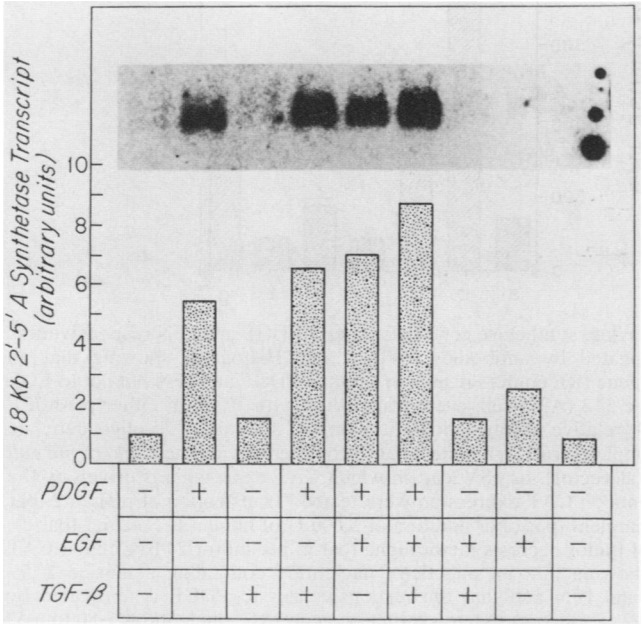

FIG. 3. Specificity of the 2',5'-oligoadenylate synthetase mRNA response to growth factors. Quiescent BALB/c 3T3 cells were treated for 9 h with the indicated growth factors under conditions similar to those described in the legend to Fig. 1. Purified recombinant PDGF (B-chain homodimer), EGF, and TGF-β were each tested at a concentration of 20 ng/ml. For further details, see Materials and Methods.

Induction of 2',5'-oligoadenylate synthetase by pure recombinant PDGF. Induction of 2',5'-oligoadenylate synthetase transcripts was accompanied by an increase in enzyme activity in quiescent BALB/c 3T3 cells treated with pure recombinant PDGF. At a concentration of 2 ng/ml, the pure PDGF preparation triggered a 4-fold increase in the level of 2',5'-oligoadenylate synthetase; at 10 ng/ml, this increase was more than 10-fold (Fig. 4). For comparison, 300 U of partially purified PDGF per ml (equivalent to about 60 ng of pure PDGF per ml; not shown) elicited a 10-fold increase, and 500 U of IFN-β per ml elicited a 20-fold increase, in the level of enzyme activity (Fig. 4).

Induction of expression of 2',5'-oligoadenylate synthetase transcripts and of 2',5'-oligoadenylate synthetase by calf serum. The experiments shown in Fig. 1 through 4 were conducted with purified or partially purified growth factors; however, many laboratories manipulate cells in culture by the addition or withdrawal of serum. To determine whether the responses to PDGF shown in Fig. 1 through 4 were also elicited by calf serum, 2',5'-oligoadenylate synthetase mRNA and enzyme levels were measured in serum-stimulated cells.

Incubation with calf serum at a concentration of 10 to 40% (vol/vol) induced the 1.8- and 4-kb 2',5'-oligoadenylate synthetase RNAs (data not shown). (Using radioreceptor-binding assays, we have shown previously that 10% calf serum-supplemented medium contains between 1.5 and 5 ng of PDGF per ml [41].) Calf serum also increased the level of 2',5'-oligoadenylate synthetase. Exposure of quiescent cells to 20% calf serum caused a 3.5-fold increase in the level of the 2',5'-oligoadenylate synthetase within 4 h.

IFN and PDGF act on a 40-bp upstream element of the 2',5'-oligoadenylate synthetase gene. Recently, we (36) and others (9) have confirmed the significance of 5' upstream elements of the human 2',5'-oligoadenylate synthetase gene in conferring IFN responsiveness on the gene. Our recent data show that an element comprising nucleotides −113 to −74 (relative to a presumed translational start site) of a

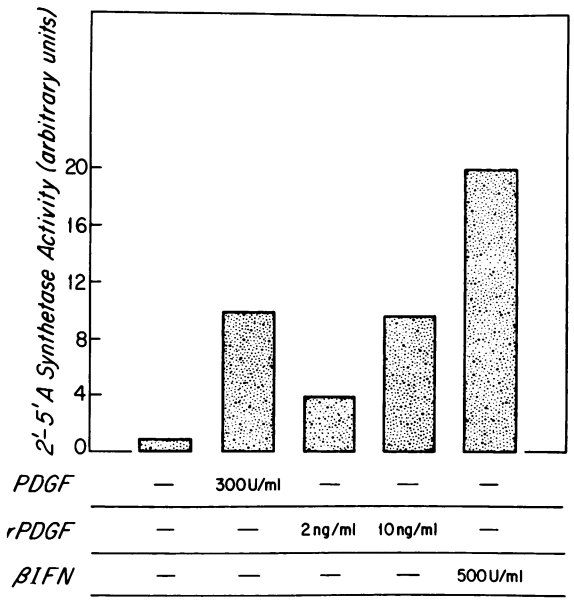

FIG. 4. Induction of 2',5'-oligoadenylate synthetase by recombinant PDGF. Quiescent BALB/c 3T3 cells were treated for 9 h with partially purified PDGF, purified recombinant PDGF (rPDGF), or murine IFN-β at the doses indicated. Cytoplasmic 2',5'-oligoadenylate synthetase was tested by the poly(rI · rC) agarose assay. The relative amounts of 2',5'-oligoadenylate synthetase activity are plotted versus the agent used to induce the cells. For further details, see Materials and Methods.

human 2',5'-oligoadenylate synthetase gene acts as an IRE. Moreover, this element binds IFN-induced nuclear factors from a variety of IFN-responsive cells (36).

We tested the ability of this element to confer responsiveness to PDGF on a heterologous promoter. For this purpose, we used a plasmid (pBLcat2) in which an enhancerless herpesvirus thymidine kinase promoter was driving CAT expression. We inserted two copies of the element comprising nucleotides −113 to −74 from the human 2',5'-oligoadenylate synthetase gene in the appropriate orientation to the 5' side of the herpesvirus thymidine kinase promoter in pBLcat2, thereby generating a new plasmid, p(Alu)FF. Construction of p(Alu)FF has been described elsewhere (25, 36). Transfection of pBLcat2 (lacking an inserted IRE) resulted in low CAT activity (Fig. 5A, bar a), whereas transfection of pSV2cat (in which CAT expression is driven by the simian virus 40 early promoter and enhancer) resulted in higher CAT activity (bar b). Transfection of p(Alu)FF (with the IRE from a human 2',5'-oligoadenylate synthetase gene) (bars c to g) resulted in higher CAT activity (bar c), which was further boosted by treatment with IFN-α2/α1 (bar d) or partially purified PDGF (bar e) but not by EGF (bar f) or tumor necrosis factor alpha (bar g). Similar results were obtained with constructs including a mouse 2',5'-oligoadenylate synthetase IRE (Fig. 5B, bars a to c). These results indicate that the IRE-containing segments from the human or mouse 2',5'-oligoadenylate synthetase gene can confer responsiveness to PDGF on a heterologous promoter.

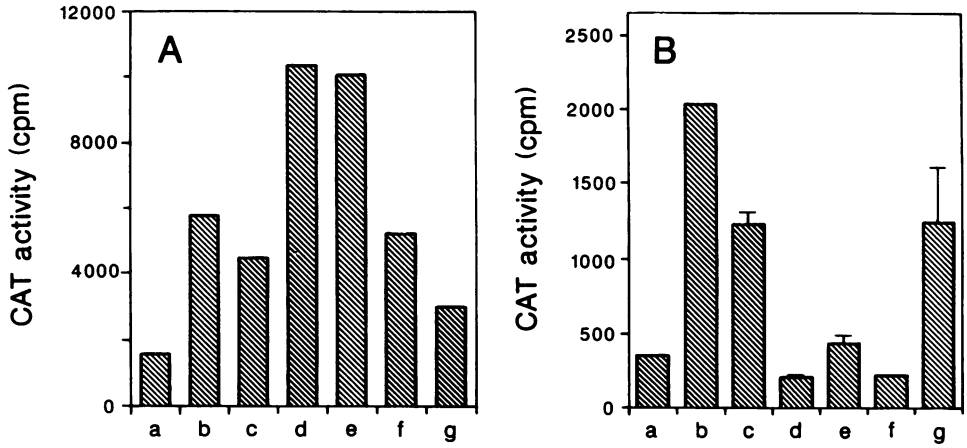

FIG. 5. Evidence that oligodeoxynucleotides containing 2',5'-oligoadenylate synthetase gene IREs confer PDGF and IFN responsiveness to a heterologous promoter and that the response to PDGF is abrogated by antibodies to IFN. (A) Histogram showing that an oligodeoxynucleotide containing a human 2',5'-oligoadenylate synthetase gene IRE confers responsiveness to PDGF and IFN but not to EGF or tumor necrosis factor alpha. For this assay, 100-mm plates of BALB/c 3T3 (A31) cells were transfected with 10 μg of either p(Alu)FF (containing two copies of the fragment spanning nucleotides −113 to −74 [relative to translation initiation]) of a human 2',5'-oligoadenylate synthetase gene cloned in the forward orientation relative to the herpes simplex virus thymidine kinase promoter driving the *Escherichia coli cat* structural gene in pBLcat2 (9, 36), pBLcat2 (the enhancerless parental vector), or pSV2cat (in which CAT expression is driven by the simian virus 40 early promoter and enhancer). The effects of several agents on CAT expression were tested. Transfection of pBLcat2 (bar a), pSV2cat (bar b), and p(Alu)FF (bars c to g) was followed by mock treatment (bar c) or addition of 2,000 U of human IFN-α2/α1 (bar d), 100 U of partially purified PDGF (bar e), 20 ng of EGF (bar f), or 20 ng of tumor necrosis factor alpha (bar g) per ml for 24 h. Cellular CAT activity was measured as described previously (24). (B) Histogram showing that an oligodeoxynucleotide containing a murine 2',5'-oligoadenylate synthetase gene IRE confers responsiveness to PDGF and IFN and that the responsiveness to PDGF is abrogated by antibodies to murine IFN. A 29-bp oligonucleotide containing the murine 2',5'-oligoadenylate synthetase gene IRE, nucleotides −80 to −52 (relative to translation initiation), was cloned in the forward orientation upstream of the thymidine kinase promoter in pBLcat2 (see above). This recombinant plasmid was transfected into cells as described for panel A). Transfections were followed by mock treatment (bar a) or addition of 2,500 U of human IFN-α2/α1 (bar b) or 200 U of partially purified PDGF (bars c to g) per ml. The cells were also exposed to 200 neutralizing units of monoclonal rat anti-mouse IFN-β (7F-D3) (bar d), polyclonal rabbit anti-mouse IFN-α/β (bar e), polyclonal sheep anti-mouse IFN-α/β (bar f), or polyclonal calf anti-human IFN-β (bar g) per ml. Data are from a representative experiment. Error bars indicate variation about a mean of duplicate transfections. All points are corrected for counts per minute obtained from cells transfected with pBLcat2 and treated as above (340 to 360 cpm). For further details, see Materials and Methods.

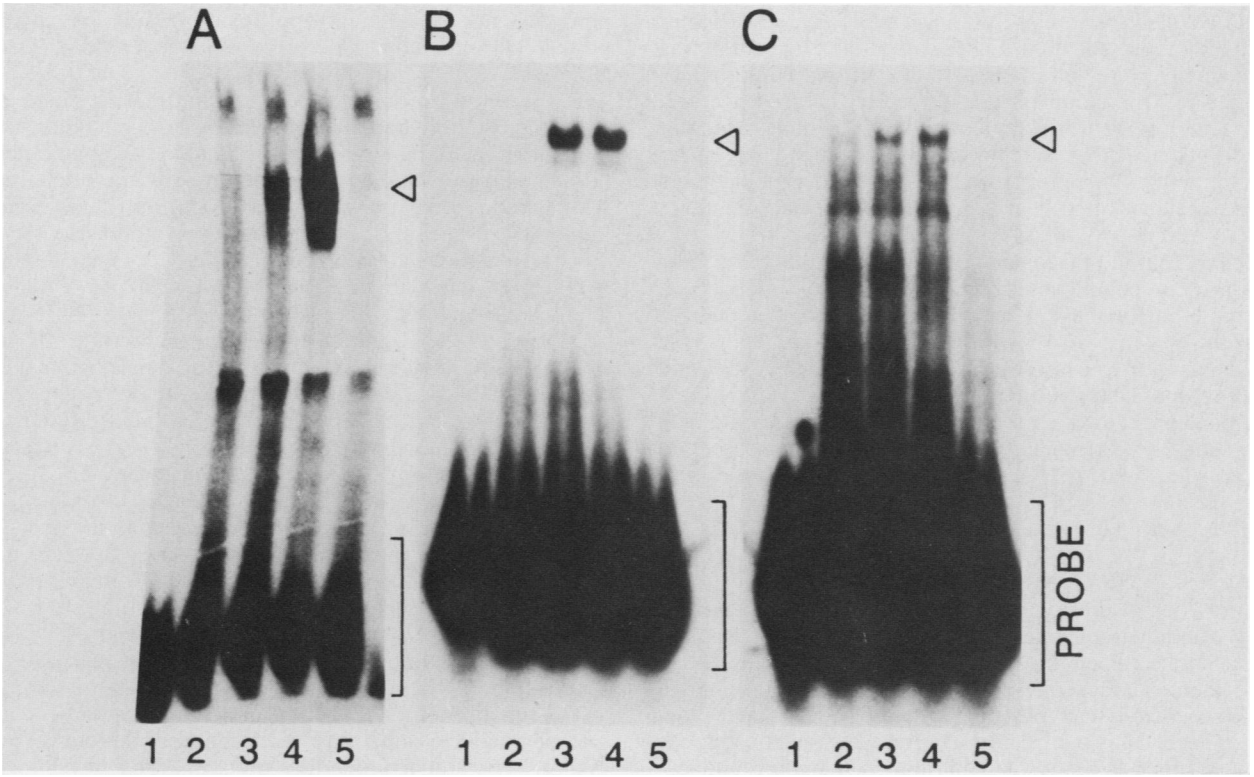

FIG. 6. Regulation of DNA-binding nuclear factors by IFN and PDGF. An end-labeled oligodeoxynucleotide (10,000 cpm, 20 pg) containing a murine IRE was incubated without nuclear extract (lane 1) or with 5 μg of nuclear extract protein from quiescent cells (lane 2) or from quiescent cells treated for 30 min with 100 U of partially purified PDGF (lane 3) or 1,000 U of human IFN-α2/α1 (lanes 4 and 5) per ml. The binding reaction shown in lane 5 also included 0.1 μg of unlabeled oligodeoxynucleotide containing the murine IRE. The incubated reaction mixtures were analyzed by band shift assays. (B) The labeled oligodeoxynucleotide used in panel A was incubated without nuclear extract (lane 1) or with 5 μg nuclear extract protein from quiescent cells (lane 2) or from quiescent cells treated for 20 min with 1,000 U of human IFN-α2/α1 per ml (lanes 3 to 5). The binding reaction in lane 4 also included 0.2 μg of an oligodeoxynucleotide spanning the serum-responsive element of c-*fos*, a nonspecific competitor (of the sequence 5'-GATGTCCATATTAGGACATC-3'); the binding reaction in lane 5 also included 0.2 μg of the oligodeoxynucleotide containing the murine IRE, a specific competitor (of the sequence 5'-CCCTTCTCGGGAAATGGAAACTGAAAATC-3'). This is the sequence spanning nucleotide positions −80 to −52 (relative to the translational start site) of a murine 2',5'-oligoadenylate synthetase gene (9). (C) The labeled oligodeoxynucleotide used in panel A was incubated without nuclear extract (lane 1) or with 10 μg of nuclear extract protein from quiescent cells (lane 2) or from quiescent cells treated for 20 min with 200 U of partially purified PDGF per ml (lanes 3 to 5). The binding reaction in lane 4 also included the nonspecific competitor (as in panel B, lane 4), and the binding reaction in lane 5 also included the specific competitor (as in panel B, lane 5). Autoradiography was twofold longer for panel C than for panel B. Tris acetate native gels (4) were used in panel A, whereas Tris glycine native gels (40) were used in panels B and C. Arrowheads indicate positions of the labeled oligodeoxynucleotide bound to protein, and the probe indicates the position of the unbound oligodeoxynucleotide. For further details, see Materials and Methods.

It is conceivable that PDGF (and also serum) requires IFNs in the medium to induce expression of the 2',5'-oligoadenylate synthetase gene(s). We tested this possibility by including anti-mouse IFN antisera in the medium surrounding the transfected cells during induction with PDGF. Enhancement by PDGF of CAT activity in cells transfected with the construct containing a murine IRE (compare bars c and a in Fig. 5B) was abrogated by a rat monoclonal anti-mouse IFN-β antibody (bar d), by a rabbit polyclonal anti-mouse IFN-α/β antiserum (bar e), or by a sheep polyclonal anti-mouse IFN-α/β antiserum (bar f) but not by a polyclonal calf anti-human IFN-β antiserum (bar g). Treatment with mono- or polyclonal anti-mouse IFN antibodies also inhibited the induction by PDGF of CAT expression from plasmid p(Alu)FF containing the human IRE. This experiment has been repeated six times with the same results (not shown). Abrogation of the PDGF effect by anti-mouse IFN antisera indicates that activation by PDGF of a heterologous promoter driven by an IRE-containing segment from a human or murine 2',5'-oligoadenylate synthetase gene requires IFN in the medium. (Treatment with an anti-mouse IFN antiserum also impaired the activation of endogenous 2',5'-oligoadenylate synthetase genes in BALB/c 3T3 cells by PDGF. The extent of impairment in this case was 75% in two experiments [not shown].) The lack of abrogation of the effect of PDGF by the anti-human IFN-β antiserum is not unexpected in view of the species specificity of IFN-β.

PDGF and IFNs may induce the binding of a common *trans*-acting factor(s). We synthesized an oligodeoxynucleotide containing the murine IRE and tested for binding of PDGF- and IFN-induced nuclear factors to this oligodeoxynucleotide by performing band shift assays.

Incubation of the labeled oligodeoxynucleotide with nuclear extracts from cells treated with either partially purified PDGF or human IFN-α1/α2 resulted in the formation of specific complexes (Fig. 6A, lanes 3 and 4). The specificity of these complexes is revealed by the fact that they were not detected in incubation mixtures including an excess of the same but unlabeled oligodeoxynucleotide (lane 5). The specificity of the complexes was further verified by using a

second gel electrophoresis system, which resulted in tighter bands than those shown in Fig. 6A. Complex formation was IFN inducible (compare lanes 2 and 3 in Fig. 6B) and was specific; i.e., the complex was formed in the presence of a nonspecific competitor oligodeoxynucleotide (lane 4) but not in the presence of a specific competitor oligodeoxynucleotide (lane 5). Complex formation was PDGF inducible (compare lanes 2 and 3 in Fig. 6C) and specific (compare lanes 4 and 5).

The fact that the PDGF-induced and IFN-induced specific DNA-protein complexes comigrated in two different gel systems (compare lane 3 with lane 4 in Fig. 6A; compare lanes 3 and 4 in Fig. 6B with lanes 3 and 4 in Fig. 6C) is consistent with the interesting possibility that PDGF and IFNs may induce (directly or indirectly) the activation of the same nuclear factor(s). An oligodeoxynucleotide containing the human 2′,5′-oligoadenylate synthetase IRE also bound such PDGF- and IFN-activatable nuclear factor(s) (not shown).

DISCUSSION

In previous studies, we have shown that partially purified PDGF stimulates expression of a 1.8-kb mRNA transcript which is recognized by a synthetic oligodeoxynucleotide probe identical in sequence to a segment from a human 2′,5′-oligoadenylate synthetase gene (51). In studies described here, using a probe for a murine 2′,5′-oligoadenylate synthetase gene, we have confirmed this finding and extended it by showing that (i) pure recombinant PDGF (the B-chain homodimer) induces a 2′,5′-oligoadenylate synthetase enzyme(s) as well as two 2′,5′-oligoadenylate synthetase mRNAs (of 1.8 and 4 kb), (ii) the time courses of the induction processes mediated by PDGF and by IFN-β are similar, and (iii) of several serum growth factors known to affect proliferation of BALB/c 3T3 cells, only PDGF modulates expression of the 2′,5′-oligoadenylate synthetase gene(s) in these cells.

We have identified a 40-bp region which is located upstream of the 2′,5′-oligoadenylate synthetase promoter, is required for activation of this gene by IFN in vivo (see also reference 9), and can bind IFN-modulated factors in vitro (36). Transient transfection assays with BALB/c 3T3 cells revealed that this 40-bp region is also responsive to PDGF, and band shift experiments indicated that it binds nuclear factors which are modulated by PDGF. The nuclear factors binding to the 40-bp region are modulated by PDGF and IFN within the same 20-min time span.

The following observations make it likely that induction of 2′,5′-oligoadenylate synthetase by PDGF is not secondary to induction by PDGF of an endogenous IFN: (i) PDGF and IFN-β modulate this nuclear binding activity with similar kinetics, (ii) PDGF and IFN-β stimulate induction of 2′,5′-oligoadenylate synthetase mRNAs and protein with comparable kinetics, and (iii) PDGF induces 2′,5′-oligoadenylate synthetase mRNA in the presence of drugs that inhibit protein synthesis by over 99% (51; data not shown).

Paradoxically, however, the PDGF response of gene constructs containing this 40-bp region was inhibited in transfected BALB/c 3T3 cells by mono- and polyclonal antibodies to mouse IFN. These data suggest that endogenous IFN in the culture medium is needed to render the gene construct responsive to PDGF. The inhibitory action of IFN antibodies is consistent with previous data showing that induction of 2′,5′-oligoadenylate synthetase in various cell lines by agents other than IFN (e.g., colony-stimulating factor I, phorbol ester, or dimethyl sulfoxide) could be at least partially dependent on the action of endogenous IFN (12, 32, 50).

We have recently demonstrated that this 40-bp region from the 2′,5′-oligoadenylate synthetase gene binds IFN-modulated factors in extracts from monkey and human cells (36). This factor binding is induced by IFN in human cells with kinetics that are similar to those for transcriptional induction of the 2′,5′-oligoadenylate synthetase gene. A comparison of the 40-bp region from the 2′,5′-oligoadenylate synthetase gene with the 5′ upstream flanking regions of other IFN-regulated genes, i.e., the 6-16 gene (30) (kindly provided by I. Kerr and G. Stark) and the murine 202 gene (N. Ito, G. Gribaudo, E. Toniato, A. Thakur, Y. Yagi, J. Barbosa, M. Kamarck, F. H. Ruddle, and P. Lengyel, *in Proceedings of the UCLA Symposia Conference on Growth Inhibitory and Cytotoxic Peptides*, in press), reveals a shared sequence (GGAAA-GAAA) which may be a recognition element for IFN-modulated nuclear factors. The functional significance of such a shared sequence is consistent with the fact that in gel retardation assays, the 6-16 gene element is able to compete with the 2′,5′-oligoadenylate synthetase 40-bp region for factor binding (not shown). It remains to be seen whether the 6-16 gene and other IFN-activatable genes containing the shared sequence are also inducible by PDGF or other growth factors.

The function of the induction of 2′,5′-oligoadenylate synthetase by PDGF remains to be established. It is conceivable that it is part of a protective program. The physiological scenario when the PDGF response comes under strong evolutionary selection pressure is the recovery from injury. PDGF released when platelets aggregate during blood clotting has many physiological functions (35). Several of these, including the mitogenic stimulus on fibroblasts, are thought to be involved in wound healing and the reinsulation of the organism from foreign pathogens. Therefore, it seems advantageous for PDGF to increase the level of 2′,5′-oligoadenylate synthetase and thus prime the 2′,5′-oligoadenylate system. This system could establish in the PDGF-stimulated cells a latent antiviral state. Alternatively, the 2′,5′-oligoadenylate system might mediate a feedback inhibition of cell division during wound healing, as suggested in the case of liver regeneration (43). This response may be transient, thus minimizing a long-term antiproliferative effect.

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