Evidence for a Novel Signal Transduction Pathway Activated by Platelet-Derived Growth Factor and by Double-Stranded RNA

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Platelet-derived growth factor (PDGF) and the synthetic double-stranded RNA poly(I) · poly(C) [poly(I · C)] stimulate transcription of the JE gene in BALB/c-3T3 fibroblasts. The response of JE to poly(I · C) does not appear to be channeled through any known component of the PDGF receptor signal transduction apparatus. In addition, JE sequences upstream of the transcription start site are devoid of previously identified poly(I · C)-responsive elements, such as those found in the beta-interferon gene. These data suggest that a novel signal transduction pathway regulates the JE response to PDGF and double-stranded RNA. The *c-myc* and *c-fos* proto-oncogenes also respond to this pathway but with poor efficiency. However, this pathway operates very efficiently on other PDGF-inducible genes that encode the secretory proteins KC and M-CSF.

Platelet-derived growth factor (PDGF) stimulates expression of a number of genes in quiescent BALB/c-3T3 fibroblasts. These PDGF-responsive genes have been described as competence genes after the term defined elsewhere to characterize the mitogenic response of BALB/c-3T3 cells to PDGF (37). The proto-oncogenes *c-myc*, *c-fos*, and *c-jun* are part of this competence gene family (16, 24, 25, 27, 32, 38, 40, 45). Their products are localized to the nucleus (25, 32, 39, 41). The *c-fos* and *c-jun* proteins may function in concert as regulators of transcription (28, 41, 48). Several other PDGF-inducible gene products, including JE and KC, are cytokinelike proteins that are secreted from the cell (9, 43; P. Oquendo et al., *J. Biol. Chem.*, in press). The M-CSF gene, encoding a well-characterized cytokine, is also induced by PDGF in BALB/c-3T3 cells (43).

Induction of the competence genes by PDGF occurs in the presence of cycloheximide (CHX), suggesting that the signaling proteins exist within the cell (9, 15, 24, 25, 32). However, the various competence genes do not respond coordinately to a common intracellular second signal. Induction of the *c-myc* and *c-fos* genes by PDGF is channeled, at least in part, through the hydrolysis of phosphatidylinositol (PI) bisphosphate and the activation of protein kinase C (10, 18, 22, 29), yet the JE and KC genes do not appear to use protein kinase C as an intermediate in the induction process (18). Rather, they respond to an alternate intracellular second messenger that is generated in response to PDGF (18). Components of the signal transduction pathway that mediate the response of JE and KC to PDGF remain to be identified.

Several years ago, it was observed that the synthetic double-stranded RNA poly(I) · poly(C) [poly(I · C)], a potent inducer of beta-interferon, stimulates competence gene expression (56). The response of the beta-interferon gene to poly(I · C) has been well characterized (12-14, 55). The promoter sequences necessary for induction of beta-interferon by poly(I · C) are known and include a negatively regulated enhancer element (12-14, 55). Activation of the enhancer by poly(I · C) is thought to be channeled through an intracellular protein kinase that is dependent upon inter-

nalized poly(I · C) (30, 31, 33, 35, 36). Recent observations provide circumstantial evidence that this double-stranded, RNA-dependent protein kinase can also respond to serum growth factors (54).

The overlap between the PDGF, serum, and poly(I · C) studies suggested to us that the response of competence genes to poly(I · C) could help identify components of that other branch of the PDGF signal transduction pathway—the branch that accounts almost totally for JE and KC gene induction by PDGF and which is partially accountable for *c-myc* gene induction (18). Accordingly, we have explored the molecular basis of competence gene induction by poly(I · C).

MATERIALS AND METHODS

Cell culture, growth factors, and reagents. BALB/c-3T3 fibroblasts (clone A31) were maintained in Dulbecco modified Eagle medium supplemented with 10% bovine calf serum. All experimental procedures were initiated on confluent density-arrested monolayer cultures that had been incubated overnight in Dulbecco modified Eagle medium supplemented with 5% platelet-poor plasma. Cells were treated with poly(I · C) at concentrations ranging from 0.001 to 50 µg/ml, partially purified PDGF (300 U/ml corresponding to 50 ng/ml), or serum (30%) for the times indicated in the Results section.

Human defibrinogenated platelet-poor plasma and partially purified PDGF (predominantly the A:B isoform of PDGF) (19) were prepared as described (1, 37). Polyinosinate-polycytidylate and polyadenosinate-polyuridinate were purchased from Pharmacia AB, Biotechnology. Polyinosinic acid, polycytidylic acid, polyguanylic-inosinic acid, dextran sulfate, polyvinyl sulfate, 12-O-tetradecanoylphorbol 13-acetate (TPA) and cycloheximide were purchased from Sigma Chemical Co. Nuclease S1 and restriction enzymes were from Boehringer Mannheim Biochemicals.

RNA extraction, electrophoresis, nuclear runoff transcription assay, S1 nuclease assay, and protein phosphorylation. Total RNA was isolated by the guanidinium isothiocyanate method, purified on CsCl gradients, and quantitated as described (9). Total RNA was electrophoresed on formalde-

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TABLE 1. Expression of the JE gene is not affected by other polynucleotides or polyanions^a

Inducing agent	Gene expression [% induction by poly(I · C)]
Poly(I · C)	100
Poly(I · C) (boiled in 1 M NaOH)	1.9
Poly(A · U)	2.5
Poly(G)	9.6
Poly(I)	<0.5
Poly(C)	<0.5
Dextran sulfate	<0.5
Polyvinyl sulfate	<0.5

^a Cells were stimulated for 2 h with 50 µg of each agent per ml, and RNA was analyzed by Northern (RNA) blot as shown in Fig. 4 (probed with JE cDNA). The films were scanned with a densitometer, and values were compared with those of poly(I · C) induction. All nucleotides were in the ribose form.

hyde gels, transferred to nitrocellulose, and probed as described (18). Insert probes were from the following genes: pJE and pKC (9), pMc-myc (46), mouse *c-fos* (11), human macrophage colony-stimulating factor (M-CSF; 53), mouse beta-interferon (21), and mouse beta-actin. Nuclear runoff transcription reactions were done according to the method of Greenberg and Ziff with modifications (18). Radiolabeled runoff RNA was hybridized to linearized DNA (10 µg) bound to nitrocellulose. Genomic DNA from BALB/c fibroblasts (0.1 µg) was used to standardize the transcriptional level. Cellular phosphoproteins were displayed as previously described (18). S1 nuclease analyses were performed as previously described (50) with minor modifications. Total RNA (20 µg) was hybridized to 0.5 µg of end-labeled probe at 48°C in 20 µl of 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4), 400 mM NaCl, 1 mM EDTA, and 80% formamide. S1 digestion was performed by adding 300 µl of buffer containing 280 mM NaCl, 30 mM sodium acetate, 4.5 mM ZnCl₂, 20 µg of salmon sperm DNA (denatured) per ml, and 500 U of S1 nuclease per ml. Samples were incubated at 37°C for 30 min, precipitated, and loaded on a 6% denaturing polyacrylamide gel (7 M urea). The probe for *c-myc* was end labeled at the *Xho*I site in the first exon, 356 bases downstream of the P2 promoter. The JE probe was end labeled at an *Nae*I site 127 bases downstream of the promoter.

RESULTS

PDGF and poly(I · C) stimulate transcription of JE at a common site. The JE gene, first isolated and characterized as a PDGF-responsive gene, is induced very well by poly(I · C) (56). As presented in Table 1, poly(A · U), poly(I), poly(C), or poly(G) do not induce the expression of JE to the level attained by poly(I · C). When poly(I · C) is hydrolyzed by boiling in 1 M NaOH, it is ineffective at inducing expression of JE (Table 1). These data show that the response of JE is specific to poly(I · C) and not to random polynucleotides. This specificity is similar to the induction of beta-interferon by synthetic double-stranded RNA (47).

Nuclear runoff transcription analysis (Fig. 1A) shows that induction of JE mRNA by poly(I · C) results from increased initiation of JE gene transcription, as is the case for PDGF-mediated JE induction (18). These runoff data show that PDGF induces a somewhat more rapid transcriptional response than does poly(I · C). When mRNA levels were assayed, it was found that there was a slight delay (approx-

FIG. 1. Poly(I · C) and serum enhance transcription of the JE gene at a common start site. (A) Nuclear runoff transcription assay. Cells were stimulated for 30, 60, or 120 min (Min's) with poly(I · C) (50 µg/ml) or PDGF (50 ng/ml). Q, Quiescent cells. Nuclei were isolated and a nuclear runoff transcription assay was performed as described in Materials and Methods. The negative control of the vector alone showed no hybridization (data not shown). (B) S1 nuclease protection assay. Cells were stimulated with 25% bovine calf serum (BCS) or with poly(I · C) (50 µg/ml) for 2 h, and RNA was isolated and analyzed by an S1 nuclease protection assay. The location of the end-labeled probe is also shown (×). The sizes of the molecular weight markers (in bases) are also shown.

imately 30 min) in the accumulation of JE transcripts by poly(I · C), compared with induction by PDGF (data not shown). Also, data indicate that JE protein is made and secreted from cells stimulated with poly(I · C) (B. J. Rollins, manuscript in preparation).

We analyzed RNA isolated from cells treated with either poly(I · C) or PDGF to determine if the start site for transcription of the JE gene was the same. An S1 nuclease protection assay was performed by using an end-labeled fragment that spans the first exon of the JE gene and extends 414 bases 5' of the previously mapped PDGF-inducible start site as a probe. The data in Fig. 1B show no qualitative difference in the protected fragments, suggesting that the start site for transcription is the same for transcripts induced by both agents.

Poly(I · C) does not activate protein kinase C. The mechanism by which double-stranded RNA regulates gene expres-

FIG. 2. Poly(I · C) is not capable of activating protein kinase C. Cells were prelabeled with $^{32}\text{PO}_4$ for 2 h and then were treated for 10 min with 12-O-tetradecanoylphorbol 13-acetate (TPA; 50 ng/ml) or PDGF (50 ng/ml), or for 10 or 30 min with poly(I · C) (50 $\mu\text{g}/\text{ml}$). Proteins were extracted with trichloroacetic acid, solubilized in an SDS loading buffer, and electrophoresed on a 12% SDS-polyacrylamide gel. The autoradiogram is shown. p80 is a substrate of protein kinase C.

sion is poorly understood. One pathway used by growth factors and oncogenes to exert an effect on gene expression involves the stimulation of cellular PI turnover. Increased turnover of PI and its phosphorylated derivatives PIP and PIP₂ has been implicated in cellular responses to a wide variety of stimuli, including growth factors such as PDGF (for reviews, see references 3 and 34). Turnover of PIP₂ results in the accumulation of diacylglycerol (DAG), which activates protein kinase C, and inositol triphosphate, which mobilizes intracellular calcium (3, 34).

A measure of the activation of protein kinase C by DAG is the extent of phosphorylation of intracellular protein substrates. One protein kinase C substrate, an 80,000-dalton cytoplasmic protein, has been well characterized (4, 44). As seen in the sodium dodecyl sulfate (SDS)-polyacrylamide gel of cellular phosphoproteins in Fig. 2, poly(I · C) did not induce the phosphorylation of this protein while both PDGF and the phorbol ester TPA did. Also, seen in this figure is a set of proteins of 31 to 32 kilodaltons (kDa) that are phosphorylated in response to PDGF and TPA. It is likely that they are the proteins associated with the small ribosomal subunit (i.e., S6 [18]). Again, poly(I · C) did not induce the phosphorylation of these proteins.

We have previously shown that the activation of protein kinase C by PDGF or the phorbol ester TPA does not

TABLE 2. Down-regulation of protein kinase C does not inhibit induction of JE by poly(I · C)^a

Gene	% Serum or poly(I · C) induction with or without pretreatment			
	Without pretreatment		With pretreatment	
	BCS	Poly(I · C)	BCS	Poly(I · C)
JE	100	420	90	575
c-myc	100	<1	45	<1

^a Quiescent 3T3 cells were left untreated or were pretreated for 24 h with TPA (50 ng/ml). Bovine calf serum (BCS) (25% [vol/vol]) or poly(I · C) (50 $\mu\text{g}/\text{ml}$) was then added to the cultures for 2 h. Total RNA was isolated, electrophoresed, blotted onto nitrocellulose, and probed with nick-translated JE or c-myc insert as described in Materials and Methods. Exposed autoradiograms were scanned with a densitometer. The results of two (c-myc) or four (JE) experiments were averaged and are presented here in arbitrary units (i.e., percentages).

contribute to the induction of the JE gene. It is still possible that poly(I · C) induces JE by only minimally activating protein kinase C. To determine if this is the case, cells were treated with TPA for 24 h (under these conditions, protein kinase C activity drops significantly [18]). At this time, the cells were stimulated with serum or poly(I · C). As seen in Table 2, JE was still able to be maximally induced by either serum or poly(I · C) after downregulation of protein kinase C. This indicates that if any protein kinase C is activated by poly(I · C), it is not responsible for induction of the JE gene. As an internal control, the c-myc gene could not be optimally induced by serum after down-regulation of protein kinase C (18).

Poly(I · C) does not activate the PDGF receptor or the associated type I PI kinase. The kinase responsible for phosphorylating PI to PIP, an 85-kDa protein, and the PDGF receptor are known to be coimmunoprecipitable with antiphosphotyrosine antibodies (23, 51). This 85-kDa protein and PI kinase activity appear to associate rapidly with the receptor after PDGF binding. This suggests that this protein and PI kinase activity are substrates of the PDGF receptor tyrosine kinase (23, 51). The activation of the receptor kinase and the association of the 85-kDa protein with the PI kinase can be demonstrated by using antiphosphotyrosine antibodies (23). To determine if these events can be induced by poly(I · C), antiphosphotyrosine immunoprecipitates were prepared from cells treated for 10 min with poly(I · C). These were then compared with immunoprecipitates from cells treated with PDGF (for 1 min or for 5 min) or with a panel of other growth factors. All immunoprecipitates were then incubated in vitro with [γ - ^{32}P]ATP, and the resulting phosphoproteins were resolved by SDS-polyacrylamide gel electrophoresis. Figure 3 (top panel) demonstrates that PDGF, but not poly(I · C), induces the autophosphorylation of the PDGF receptor (small arrow in the top left of the figure) and phosphorylation of the 85-kDa protein (large arrowhead in the middle right of the figure). Only immunoprecipitates from PDGF-treated cells showed enhanced PI kinase activity (Fig. 3, bottom panel). Thus, PDGF treatment, and not poly(I · C) treatment, results in the activation of the PDGF receptor tyrosine kinase and in the phosphorylation of PDGF receptor substrates.

Poly(I · C) does not efficiently stimulate the turnover of PI. From the results presented, it is clear that poly(I · C) has little or no effect upon protein kinase C, the PDGF receptor, or the PDGF receptor-associated PI kinase. Yet it is possible that poly(I · C) stimulates PI turnover via another unknown mechanism. To determine if this was occurring, PI turnover

FIG. 3. Poly(I · C) is not capable of activating the PDGF receptor and the type 1 PI kinase. Activation of PI kinase activity (top panel). Quiescent Balb/c-3T3 cells were treated for 10 min with β -transforming growth factor (β -TGF; 1 μ g/ml), epidermal growth factor (EGF; 100 ng/ml), or poly(I · C) (50 μ g/ml) or for 0, 1, or 5 min with PDGF (50 ng/ml). Proteins were extracted and immunoprecipitated with antiphosphotyrosine antibodies, and in vitro kinase assays were performed as described (23). Phospholabeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis. The large arrowhead on the right of the figure points to the 85-kDa protein associated with PI kinase. The small arrow at the top left points to the PDGF receptor (approximately 180 kDa). The small arrowhead in the middle of the figure points to the beta subunit of the insulin receptor (approximately 90 kDa). PI kinase assays assayed in antiphosphotyrosine immunoprecipitates are shown in the bottom panel. PI kinase assays were performed as described (23). Reactions were extracted, spotted onto thin-layer chromatographic plates, and developed as described (49, 52). Shown is a thin-layer chromatographic gel of phosphate incorporated into PI (PIP).

was measured. Figure 4 shows the results of a typical experiment where accumulations of phosphate into PI are determined (a gross measure of PI turnover). After a 15-min stimulation with serum or PDGF, an 80% increase in phosphate into PI was seen. This is comparable to that observed in previous studies with PDGF (2, 6, 20). Poly(I · C) induced only a small increase of phosphate into PI (about 20%).

Associated with PI turnover is the formation of DAG. A

measure of DAG is its phosphorylated product phosphatidic acid (PA). When cells were stimulated with PDGF or serum for 15 min, a 3.5- to 4.5-fold induction in PA levels was seen (Fig. 4), which is consistent with previous reports involving PDGF stimulation (6, 17, 20). Poly(I · C) showed only a 1.5-fold induction of PA levels. Poly(I · C) and PDGF induce similar levels of JE transcription (Fig. 1A) and JE mRNA (Fig. 5, A and B) but only PDGF is capable of dramatically increasing the levels of PI and PA. We conclude that PI and PA do not contribute to the induction of these genes by poly(I · C).

Differential response of PDGF-inducible genes to poly(I · C). PDGF and poly(I · C) may activate the same signal transduction pathway to the same extent. If this is true, then the JE, *c-myc*, *c-fos*, KC, M-CSF, and beta-interferon genes should respond equally to PDGF and poly(I · C). Dose-response studies show that this is not the case. PDGF-inducible genes which encode the secretory proteins JE, KC, and M-CSF are induced by very low concentrations of poly(I · C) (Fig. 5A and B). The amount of JE and M-CSF mRNA generated in response to poly(I · C) is equivalent to that generated in response to PDGF (see Table 3). When the films are overexposed, induction of these three genes with as little as 10 ng of poly(I · C) per ml can be observed. Assuming an average length of 4 kilobases for poly(I · C), a concentration of 10 ng/ml is in the picomolar concentration range. There was little change in the levels of actin mRNA in response to poly(I · C) (Fig. 5A).

We previously reported that the *c-fos* and *c-myc* genes were induced by poly(I · C) (56). However, in our initial studies we did not compare the serum and poly(I · C) responses (56). Shown in Fig. 6A is the response of the *c-fos* gene to two different doses of poly(I · C). Expression of *c-fos* is stimulated by poly(I · C) at concentrations as low as 1 ng/ml. However, the magnitude of *c-fos* induction by poly(I · C) is much lower than the serum-mediated response (Fig. 6B).

When we analyzed *c-myc* expression in response to poly(I · C) by Northern (RNA) blot hybridization, transcripts were detectable but the abundance levels were again very low relative to the serum response (Fig. 7A). An S1 nuclease protection assay was performed to detect low levels of *c-myc* mRNA. Serum induces *c-myc* transcripts that initiate primarily at the P2 promoter (Fig. 7B). *c-myc* transcripts induced by poly(I · C) are also detected at P2, but the response is very low relative to the serum-mediated response.

A summary of the response of these genes to poly(I · C) and PDGF (or serum) is presented in Table 3. It is clear that poly(I · C) and PDGF are capable of inducing the expression of the JE, KC, and M-CSF genes to nearly the same levels. Yet, poly(I · C) has only a minimal effect on induction of the *c-myc* and *c-fos* genes.

Figure 8 shows that induction of *c-myc* and *c-fos* expression by poly(I · C) increased in the presence of CHX. In the presence of serum alone, no accumulation of *c-fos* mRNA is observed because mRNA levels were assayed at 2 h post-stimulation, at which time *c-fos* mRNA transcripts typically return to base-line values (18, 25, 32).

As a frame of reference for *c-fos* and *c-myc* induction depicted in Fig. 8, the JE gene shows the expected induction by poly(I · C) and superinduction in the presence of poly(I · C) plus CHX. The lower panel shows that beta-interferon is also strongly superinduced by poly(I · C) and CHX, consistent with earlier findings (5, 42). However, no

FIG. 4. Poly(I · C) does not efficiently stimulate the turnover of PI. Accumulation of ^{32}P -labeled inorganic phosphate into PI (left panel) and phosphatidic acid (PA; right panel). Cells were prelabeled for 2 h with $^{32}\text{P}_4$ and then were stimulated for 15 min with control (▨), PDGF (50 ng/ml), 15% serum, or poly(I · C) (50 $\mu\text{g}/\text{ml}$). PI and phosphatidic acid levels were assayed as described (49, 52) by extracting the reactions, spotting them onto thin-layer chromatographic plates, and counting them in a scintillation counter.

FIG. 5. Response of the JE, KC, and M-CSF genes to poly(I · C). Quiescent BALB/c-3T3 fibroblast cells were stimulated for 2 h with various doses of poly(I · C) or with PDGF (50 ng/ml). Total RNA was isolated, electrophoresed, and blotted as described in Materials and Methods. The nitrocellulose blots were hybridized with nick-translated probes of (A) JE, KC, and mouse beta-actin (β -actin) or (B) the human M-CSF gene (the arrow points to the major 4.2-kilobase transcript).

induction of beta-interferon could be detected in response to serum or serum plus CHX (or PDGF, see Discussion).

DISCUSSION

In a previous report, we demonstrated that the synthetic double-stranded RNA poly(I · C) stimulates the expression of genes that are also responsive to PDGF (56). Dose-response studies outlined here show that various PDGF-responsive genes do not respond equally to poly(I · C). Two genes that encode intracellular proteins (*c-myc* and *c-fos*) respond well to serum or PDGF (24) but weakly to poly(I · C). Three genes that encode cytokines or cytokine-like proteins (JE, KC, and M-CSF) respond very well to PDGF and also to poly(I · C). Finally, the beta-interferon gene is induced by poly(I · C) but shows no detectable response to serum or PDGF (see below).

The JE gene which responds well to PDGF and to poly(I · C) was singled out for further studies. We observed that both PDGF and poly(I · C) induce JE expression by stimulating transcription initiation. The JE transcripts induced by either PDGF or by poly(I · C) initiate at the same position. We found no evidence that JE induction by poly(I · C) is channeled through activation of the PDGF receptor (Fig. 3). Likewise, the response of JE to poly(I · C) does not appear to be channeled through any element of the one PDGF signal transduction pathway that has been well

TABLE 3. Summary of the responses of the JE, KC, M-CSF, *c-fos*, and *c-myc* genes to PDGF (or serum) and poly(I · C)^a

Gene	Responses (%) to PDGF (serum) or poly(I · C) induction in:			
	mRNA levels		Nuclear runoff transcription	
	PDGF ^b (serum)	Poly(I · C)	PDGF	Poly(I · C)
JE	100	110	100	91.5
KC	100	52.5		
M-CSF	100	77.5		
<i>c-myc</i> ^c	100	0.5–1	100	Not detectable
<i>c-fos</i> ^c	100	1	100	Not detectable

^a Autoradiographs (as in Fig. 1A, 2B, 5, 6, and 7) were scanned with a densitometer. The values from these experiments and others not presented were averaged.

^b The values for the PDGF-induced response were arbitrarily set at 100%.

^c Although the values for *c-fos* and *c-myc* induction by poly(I · C) appear low, they were two- to fourfold above the unstimulated control (data not shown). Serum was used in the *c-fos* and *c-myc* mRNA induction experiments instead of PDGF (these genes respond similarly to serum and PDGF).

FIG. 6. Response of the *c-fos* gene to poly(I · C). Northern (RNA) blots of total RNA probed with the mouse *c-fos* gene are shown. (A) Cells were stimulated with the indicated dose of poly(I · C) for 45 min. (B) Cells were stimulated with 25% serum or poly(I · C) (0.001 μ g/ml) for 15 min.

characterized to date—the pathway that involves hydrolysis of PI bisphosphate and the activation of protein kinase C. Because induction of JE transcription in response to poly(I · C) is somewhat delayed relative to the PDGF response (Fig. 1), we used proportionately delayed times of poly(I · C) stimulation in our search for evidence of PDGF receptor activation and protein kinase C activation. Although the negative results must be viewed with caution, no support could be garnered for the view that JE induction by poly(I · C) is channeled through activation of the PDGF receptor or protein kinase C (Fig. 2 and 3; Table 2).

How does the JE gene respond, at the transcriptional level, to PDGF and poly(I · C)? How do other genes respond selectively to PDGF or to poly(I · C)? Two plausible models are presented schematically in Fig. 9. In one model (Fig. 9A), PDGF and poly(I · C) generate distinct second messengers. The induction of individual genes by PDGF, poly(I · C), or both is dependent on transacting factors that are activated by the second messenger. In accord with this model, the JE gene responds to PDGF and poly(I · C) because it contains two elements in the promoter that are binding sites for the different factors. In a second model (Fig. 9B), PDGF and poly(I · C) each generate two second mes-

FIG. 7. Response of the *c-myc* gene to poly(I · C). Cells were stimulated for 2 h with poly(I · C) (50 μ g/ml) or PDGF (50 ng/ml). Total RNA was isolated and analyzed by (A) Northern (RNA) blot, probed with *c-myc* or (B) an S1 nuclease protection assay as described in Materials and Methods. The protected fragment corresponding to the second promoter (P2) is 356 bases long while that for the first promoter (P1) is 520 bases.

FIG. 8. Superinduction of the JE, *c-myc*, *c-fos*, and beta-interferon genes by poly(I · C) and CHX. Cells were stimulated for 2 h with 25% serum, poly(I · C) (100 μ g/ml), CHX (10 μ g/ml), serum plus CHX, or poly(I · C) plus CHX. Total RNA was isolated and analyzed by Northern (RNA) blot hybridization (as in Fig. 1) with nick-translated insert probes for JE, *c-myc*, *c-fos*, or beta-interferon.

sengers. In this model, the JE gene responds to PDGF and poly(I · C) because it contains the element that interacts with the common factor. It is also possible that a common transcription factor is activated by both PDGF and poly(I · C) and binds to the same element in the promoters of all of these genes. Further, it is possible that variations exist in the sequence for the binding site between promoters. This would result in differential binding and differential gene activation.

We favor model 2. Our bias is predicated on a nucleotide sequence analysis of the JE, M-CSF, and beta-interferon promoters. The interferon response element within the beta-interferon gene which confers responsiveness to poly(I · C) has been well characterized (12–14, 55). Neither the JE gene (43; J.-C. Irminger and C. D. Stiles, unpublished observations) nor the M-CSF gene (26), which respond to PDGF and poly(I · C), contain an interferon response element within the known 3,000 or 570 nucleotides 5' to the start site of transcription. The serum response element in the *c-fos* promoter, which renders that gene responsive to serum, has also been well characterized. However, neither the JE nor M-CSF genes contain a serum response element within the boundaries of their promoters. Since the other models predict that both JE and M-CSF promoters would contain serum response elements and interferon response elements (which they do not) and that the beta-interferon promoter contains a serum response element (which it does not), they are likely incorrect.

FIG. 9. Differential response of inducible genes to PDGF and poly(I · C). Two alternative models account for the differential response of genes to PDGF, poly(I · C), or both. (A) PDGF and poly(I · C) each generate a unique second messenger represented by a circle (●) or a box (■). The response of genes to PDGF, poly(I · C), or both is dictated by the number and nature of *cis*-acting elements upstream of the transcription start site that interact with these messengers. (B) PDGF and poly(I · C) each generate two second messengers. One is unique (● for PDGF and ■ for poly(I · C)), and one is common to both (▲). Genes such as JE, which respond to both PDGF and poly(I · C), do so because they contain the *cis*-acting element which is acted on by the common second messenger. The arrows represent the start sites for transcription.

What is the nature of the signal generated by poly(I · C) that is capable of activating gene expression? It is known that double-stranded RNA such as viral RNA or poly(I · C) is capable of activating specific protein kinases *in vivo* and *in vitro* (31, 35). One of these phosphorylates the alpha subunit of eucaryotic initiation factor two, resulting in a block in protein synthesis. Another protein kinase shows growth-related expression (36). These kinases may be activated *in vivo* by poly(I · C) and in turn generate the signal for increased gene expression. If model 2 is correct and one of these double-stranded-RNA-dependent protein kinases is involved in the signaling for increased gene expression, PDGF should also activate this kinase. Evidence for such a mechanism comes from a recent study suggesting that a double-stranded-RNA-dependent protein kinase can also be activated by serum growth factors (54).

In conclusion, double-stranded RNA induces the expression of genes coding for four secreted proteins: JE, KC, M-CSF, and beta-interferon. These proteins appear to have a role in growth regulation, differentiation, or inflammation (7, 8, 43; Oquendo et al., *in press*). It is tempting to speculate that a specific signaling pathway has evolved to mediate the

induction of these genes by double-stranded RNA (and by PDGF for the JE, KC, and M-CSF genes as well). If treatment of cells with double-stranded RNA mimics infection by viruses, as has been suggested (47), then induction of this specific family of genes may be important in mounting an immune or inflammatory response. This mechanism functions preferentially, though not quite exclusively, on genes encoding secretory proteins.

In the course of this work, we found that we could not repeat one facet of the previous study documenting overlap between PDGF-responsive and poly(I · C)-responsive genes (56). In our earlier work, we reported that PDGF would stimulate expression of the beta-interferon gene, albeit to a lesser extent than poly(I · C). In subsequent studies, a typical example of which is shown here (Fig. 8), we have been unable to observe any induction of beta-interferon whatsoever in response to serum or PDGF. The differential response of beta-interferon to PDGF and poly(I · C) appears to be qualitative rather than merely quantitative. The principle investigator (C.D.S.) deeply regrets any confusion this error caused.

ACKNOWLEDGMENTS

We thank Mariano Garcia-Blanco for critical comments.

This work was supported by Public Health Service grant CA22427 to C.D.S. from the National Institutes of Health and by Damon-Runyon grant DRG-803 to D.J.H.

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