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20. Endopeptidase assays were carried out with [^{3}H]diaminopimelic acid–labeled bisdisaccharide peptide dimer (2×10^3 cpm/mol). Determinants of transglycosylase (murein–murein-6-muramyl transferase) activity were determined with [^{3}H]diaminopimelic acid–labeled sacculi (4×10^4 cpm/μg) as a substrate.
21. Presented in part at the 90th Annual Meeting of the American Society of Microbiology, Anaheim, CA, 13 to 17 May 1990. Supported in part by NIH grants AI23459 and AI27913 (to E.T.); by a Career Investigator Award (to E.T.) from the American Lung Association; by grant DRR00480 (to Michigan State University–NIH Mass Spectrometry Facility); by an award (to S.N.) from the Washington University School of Medicine; and by the Fond National de la Recherche Scientifique, Bruxelles, Belgium (M.G.).

16 July 1990; accepted 4 October 1990

Signal Transduction by Interferon-α Through Arachidonic Acid Metabolism

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Molecular mechanisms that mediate signal transduction by growth inhibitory cytokines are poorly understood. Type I (α and β) interferons (IFNs) are potent growth inhibitory cytokines whose biological activities depend on induced changes in gene expression. IFN-α induced the transient activation of phospholipase A_2 in 3T3 fibroblasts and rapid hydrolysis of [^{3}H]arachidonic acid (AA) from prelabeled phospholipid pools. The phospholipase inhibitor, bromophenacyl bromide (BPB), specifically blocked IFN-induced binding of nuclear factors to a conserved, IFN-regulated enhancer element, the interferon-stimulated response element (ISRE). BPB also caused a dose-dependent inhibition of IFN-α–induced ISRE-dependent transcription in transient transfection assays. Specific inhibition of AA oxygenation by eicosatetraynoic acid prevented IFN-α induction of factor binding to the ISRE. Treatment of intact cells with inhibitors of fatty acid cyclooxygenase or lipoxygenase enzymes resulted in amplification of IFN-α–induced ISRE binding and gene expression. Thus, IFN-α receptor–coupled AA hydrolysis may function in activation of latent transcription factors by IFN-α and provides a system for studying the role of AA metabolism in transduction of growth inhibitory signals.

THE GROWTH INHIBITORY ACTIVITY of type I IFNs (α and β), as well as the frequent deletion of genes that encode these cytokines (on chromosome 9p22) in acute lymphoblastic leukemia (*1*), identifies these IFNs as potential tumor suppressors. Interaction of IFN-α with its membrane receptor induces transcription of genes that contain a conserved cis-acting DNA element, the interferon-stimulated response element (ISRE) (*2*). However, the IFN receptor–coupled signal transduction pathways are undefined. Conventional receptor-mediated signaling mechanisms that involve protein kinase C, cyclic adenosine monophosphate–dependent protein kinase A, or fluxes in intracellular pH or calcium, do not function in IFN-α–induced activation of ISRE-directed gene expression (*3*).

Because stimulation of phospholipase A_2 (PLA_2) is a source of second messenger for receptor-mediated signaling (*4*), we tested for activation of PLA_2 by IFN-α in mouse Balb/c 3T3 (clone A31) cells. The A31 cells were grown to confluence, labeled for 2 hours with [^{32}P]orthophosphate, and treated with IFN-α for various durations. Labeled extracts were then analyzed by thin-layer chromatography (TLC) for IFN-stimulated production of lysophospholipids, which are the products of catalysis by PLA_2 (Fig. 1A) (*5*). Within 5 min of IFN-α treatment, lysophosphatidylcholine (lysoPC) was increased by 60%, with a maximum increase of 100% after 15 min, relative to unstimulated amounts. By 2 hours after IFN treatment, lysoPC returned to near basal concentrations. Lysophosphatidylethanolamine (lysoPE) was not consistently elevated in extracts of IFN-treated cells (*6*). Cellular concentrations of phosphatidylcholine (PC) decreased by 12 to 14% within 15 min of IFN treatment, in accord with the increase in lysoPC (Fig. 1A). The transient nature of the PLA_2 response suggests that PLA_2 may participate in signaling by IFN-α.

A consequence of membrane PLA_2 activation is release of arachidonic acid (AA) from membrane phospholipids, typically PC or phosphatidylinositol (PI) (*7*). Arachidonic acid can then be used for synthesis of prostaglandins (PGs) and other eicosanoids via cyclooxygenase-, lipoxygenase-, and epoxygenase-catalyzed reactions. Because IFN-α stimulates cyclooxygenase-catalyzed PG synthesis in human fibroblasts (*8*), AA hydrolysis might be an important signaling mechanism for IFN-α. We tested directly for IFN-α–stimulated [^{3}H]AA release from labeled mouse fibroblasts. Treatment of confluent, [^{3}H]AA-labeled A31 cultures with a receptor-saturating concentration (10 ng/ml) of IFN-α led to a rapid release of [^{3}H]AA into the culture medium (Fig. 1B). Pretreatment of cultures with the phospholipase inhibitor BPB (bromophenacyl bromide) markedly inhibited both basal and stimulated [^{3}H]AA release (*6*). Treatment of A31 cells with platelet-derived growth factor (PDGF), as with IFN-α, induces binding of nuclear factors to the ISRE, albeit to a lesser degree (*9*). Similarly, PDGF (10 ng/ml) treatment of A31 cells resulted in rapid [^{3}H]AA release (Fig. 1B), as reported (*10*). Because PDGF and IFN-α exhibit antagonistic effects on fibroblast growth, AA would not be expected to act as a second messenger for both PDGF- and IFN-activated pathways. It thus seems more likely that a specific eicosanoid second messenger is generated in response to IFN-α.

To test for the participation of AA metabolism in the activation of ISRE-binding factors by IFN-α, we measured ISRE complex formation in electrophoretic mobility-shift assays (EMSA) (*11*). At subsaturating amounts of IFN-α (300 IU/ml, 60 pM), pretreatment of A31 cells in culture with nordihydroguaiaretic acid (NDGA) (10 μM), a lipoxygenase inhibitor, or indomethacin (INDO) (10 μM), a cyclooxygenase inhibitor, led to a marked amplification of the induced ISRE complex (Fig. 2A). Quantification of the relative amounts of induced ISRE-binding activity in extracts of cells pretreated with NDGA or INDO indicated that both agents yielded a threefold amplification of the induced signal, relative to nonsaturating IFN treatment (Fig. 2B). Pretreatment of A31 cells with 50 μM BW577C, an inhibitor of both lipoxygenase and cyclooxygenase enzymes, also resulted in signal amplification of approximately threefold as much as untreated cells. Another potent inhibitor of cyclooxygenase activity, flurbiprofen, had a similar amplifying effect (*6*). Amplified amounts of ISRE-binding activity were identical to those induced by a saturating dose of IFN-α (2000 IU/ml, 400 pM) in the absence of treatment with the inhibitors (Fig. 2B). In the presence of subsaturating concentrations of IFN-α, the amount of ISRE-binding activity was directly proportional to the IFN-α receptor occupancy (Fig. 2B), which regulates the rate of IFN-α–induced transcription (*12*).

Significantly, pretreatment with BPB blocked IFN-induced ISRE complex forma-

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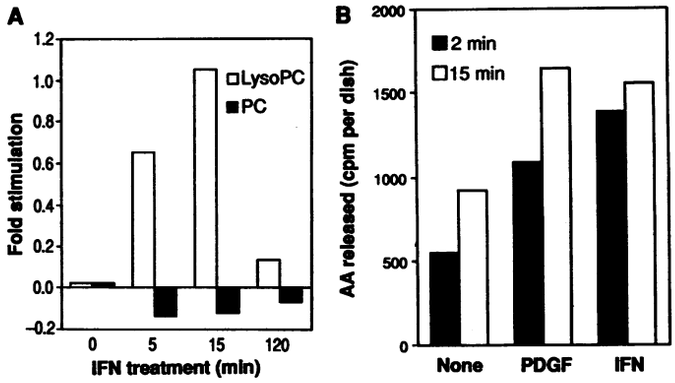

Fig. 1. IFN-α–stimulated PLA_2 activity in confluent A31 fibroblasts. (**A**) Treatment of confluent A31 cultures with a receptor-saturating concentration of IFN-α (2000 IU/ml) results in rapid stimulation of PLA_2 activity, measured as an increase in [^{32}P]lysoPC as a percentage of total labeled phospholipid (*5*). At 5 min, treatment resulted in a 65 ± 13% increase over unstimulated lysoPC concentrations, and a 105 ± 22% increase at 15 min. Bars indicate the average of duplicate plates in a representative experiment. These increases are presented as a fold stimulation value [(stimulated/unstimulated) − 1] for each time point, plotted against treatment time. There was no stimulation by IFN-α of any of the major ^{32}P-labeled lipid species (PC, PE, PI, or PS); a small decrease in PC concentration was reproducibly seen (12 ± 6% to 14 ± 3% in the experiment shown). The time course shown is representative of two independent experiments (n = 5). Additional experiments indicated that the increase in lysoPC with time is specific to the IFN-α treatment, and that the 100% increase in lysoPC at 15 min of treatment represents a maximum increase (*6*). (**B**) Stimulation of release of cellular [^{3}H]AA by human PDGF (10 ng/ml) and IFN-α (10 ng/ml). Bars indicate the average of duplicate plates in a representative experiment. In three independent experiments IFN-induced [^{3}H]AA release (measured at 2 min) was 140 ± 16% of [^{3}H]AA release without IFN treatment (*5*).

tion (Fig. 2, A and B). These changes in binding activity were relatively specific for ISRE-binding factors, because factor binding to a regulatory site from the human IFN-β gene (*13*) was unaffected by treatment with the inhibitors (Fig. 2C). In the absence of IFN, AA metabolic inhibitors did not induce detectable ISRE-binding activity (Fig. 2B). Thus, inhibition of AA metabolism through cyclooxygenase and lipoxygenase systems affected early signals generated by IFN-α binding to its receptor. This conclusion is supported by experiments in which addition of AA to intact A31 cells potentiated IFN-α induction of ISRE-binding without inducing binding in the absence of IFN (*6*).

Experiments with an analog inhibitor of AA oxygenation, 5,8,11,14-eicosatetraynoic acid (ETYA) (*14*), further indicated the participation of AA metabolism in ISRE signaling; ETYA is a specific inhibitor of AA metabolism that affects cyclooxygenase-, lipoxygenase-, and epoxygenase-catalyzed AA oxygenations (*14*). Pretreatment of A31 cultures with ETYA (10 μM) specifically inhibited subsequent IFN-α–induced ISRE binding by ~50% (Fig. 2D). This amount of inhibition is consistent with the reported effects of similar concentrations of ETYA on AA oxygenation reactions (*15*). The effects of the various AA metabolism inhibitors on IFN-α activation of ISRE-binding factors clearly implicate AA metabolism in IFN-α–induced binding of factors to the ISRE. However, formation of the putative regulatory AA metabolite does not appear to occur via cyclooxygenase or lipoxygenase reactions.

It is a formal possibility that the effect of INDO and NDGA on ISRE binding occurred because products of cyclooxygenase and lipoxygenase enzymes inhibit the binding of transacting factors to the ISRE. However, this seems unlikely, as the addition of prostaglandin E_2 (a cyclooxygenase product) to activated extracts had no effect on ISRE complex formation, nor did the compounds AA861 (a specific inhibitor of 5-lipoxygenase) or diethylcarbamazine (an inhibitor of lipoxygenase-mediated leukotriene synthesis) modify IFN-α induction of ISRE factors (*6*). A more likely interpretation is that substrate AA derived from IFN-α receptor activation is redirected from cyclooxygenase- and lipoxygenase-catalyzed transformations to another AA metabolic pathway (for example, epoxygenase-catalyzed AA conversions would not be inhibited by INDO or NDGA) (*16*). The alternate pathway may also be directly stimulated by IFN.

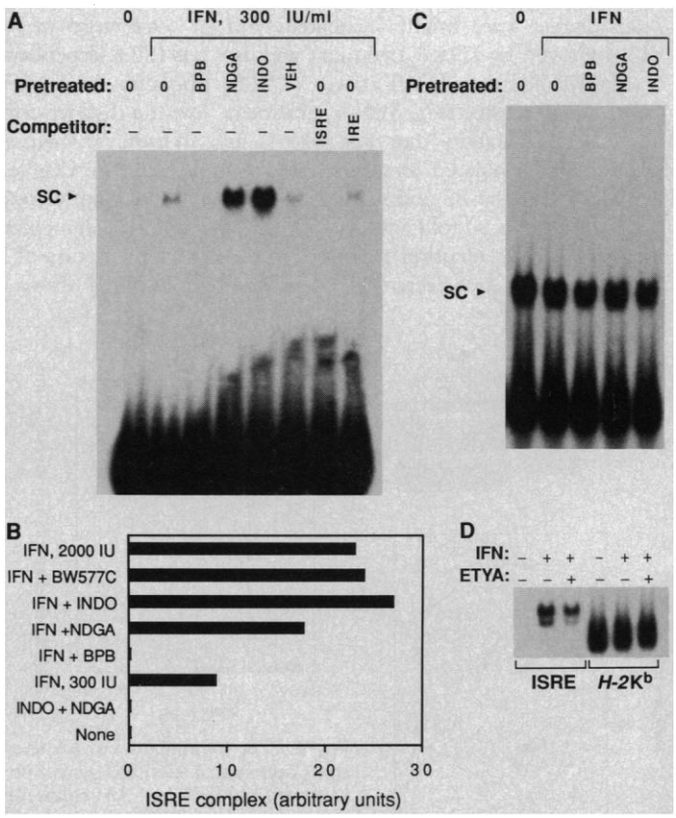

Fig. 2. IFN-α–induced ISRE binding in A31 cells exposed to inhibitors of AA metabolism. (**A**) A31 WCEs (5 μg) (*11*) were analyzed for ISRE-binding activity by EMSA (*11*). A31 monolayers were treated for 15 min with 300 IU of IFN-α per milliliter, after a 10-min pretreatment with BPB, INDO (10 μM), an inhibitor of fatty acid cyclooxygenase, NDGA (10 μM), an inhibitor of lipoxygenases or vehicle (VEH). (**B**) Autoradiograms from an experiment similar to that described in (A) were scanned by densitometer (LKB Ultrascan) to quantitate relative ISRE binding activity in WCEs from A31 cells pretreated with 10 μM BPB, 10 μM INDO, 10 μM NDGA, or 50 μM BW577C (an inhibitor of both cyclooxygenase and lipoxygenase enzymes). The results shown in (A) and (B) are representative of four independent experiments. (**C**) Whole-cell extracts in (A) were incubated with a labeled oligonucleotide representing a double-stranded RNA-inducible binding site, derived from the 5′ upstream regulatory region of the human IFN-β gene (IRE) (*11*). Specific complexes for each probe in (A) and (C) are marked SC. (**D**) Confluent A31 cultures were exposed to ETYA (10 μM) for 15 min before IFN-α treatment (600 IU/ml for 15 min). WCEs were analyzed in EMSA, as in (A). ETYA pretreatment resulted in ~50% inhibition of IFN-α–induced ISRE complex formation, as estimated by densitometric scanning of the autoradiograms. This result is representative of three independent experiments. The extracts contained equivalent amounts of binding activity specific for a synthetic oligonucleotide representing the (non-IFN–inducible) *H-2K*^{b} enhancer binding site (*11*).

In all systems studied to date, activation of ISRE-binding factors by IFN-α correlates with activation of transcription of ISRE-containing genes (*2*). In order to determine whether inhibition of factor binding to the ISRE correlated with an inhibition of IFN-induced gene expression, the effect of BPB on IFN-α induction of specific mRNA transcripts was measured. A31 cultures that had been treated with 0, 10, 30, and 50 μM BPB, were incubated with IFN-α for 4.5 hours. Total cellular RNA was isolated and analyzed (*17*) for the expression of two ISRE-containing genes, 2-5A synthetase and 1-8 (*2, 12*). IFN-induced expression of these two genes was inhibited by BPB in a dose-dependent manner, while a control actin mRNA (Fig. 3A) was unaffected by this agent.

To confirm that BPB inhibited IFN-inducible gene expression at the level of transcription, we performed transient transfection assays. A plasmid that carried the *Escherichia coli* chloramphenicol acetyltransferase (CAT) reporter gene, under the control of a viral thymidine kinase promoter and a single IFN-inducible ISRE (*2, 9*), was introduced into COS-1 (monkey) cells. Transfectants were briefly incubated with BPB followed by IFN-α treatment and assayed for IFN-induced CAT activity (*17*). In the COS-1 transfectants, IFN-α treatment induced CAT activity that was 40-fold as much as unstimulated amounts. Pretreatment with BPB at 35 and 45 μM reduced this induction to 20-fold and 4-fold, respectively (Fig. 3B). Identical results were obtained with A31 transfectants (*6*). Thus the results obtained with BPB inhibition of ISRE binding are clearly reflected at the level of IFN-α–induced ISRE-dependent transcription.

To test the general involvement of AA metabolism in IFN-α signaling, we treated L929 fibroblast cultures with natural murine IFN-α/β, in the presence and absence of cyclooxygenase and lipoxygenase inhibitors, and assayed for endogenous, IFN-induced 2-5A synthetase activity (Fig. 3C). Induction of the 2-5A synthetase gene by IFN-α is mediated by an ISRE sequence (*2*). Pretreatment of L929 cells with INDO (10 μM) or BW577C (50 μM) yielded approximately threefold increases in the amounts of induced 2-5A synthetase activity. Similar results were obtained with A31 cells (*6*). The results with L929 and COS-1 cells (Fig. 3) show that the involvement of the PLA_2/AA mechanism in IFN-α–induced gene expression is not limited to A31 cells. Furthermore, recent reports that show synergistic antitumour activity with both cyclooxygenase inhibitors and IFN-α in vivo (*18*) also indicate general involvement of this mechanism in mediating biological effects of IFN-α.

One target of IFN-α–mediated signaling of ISRE-dependent transcription is a cytoplasmic factor, ISGF3α, which associates with a distinct cytoplasmic factor, ISGF3γ, to form the ISRE-specific trans-acting factor ISGF3 (*2*). Our results suggest that generation of an eicosanoid second messenger functions in regulating the induced cytoplasmic association of ISGF3α and ISGF3γ. As mentioned above, AA is not an attractive second messenger candidate in view of the specificity that must be maintained in the IFN-α transcription response. Addition of AA to either intact cells or cell extracts does not activate ISRE-binding factors (*6*). However, AA has been shown to specifically suppress guanosine triphosphatase (GTPase)–activating protein (GAP) activity, a regulator of cellular Ras p21 GTPase activity (*19*). Treatment with IFN (type I) of c-H-*ras*–transformed 3T3 cells leads to a sustained reversion of the transformed phenotype (*20*), and thus free AA may function in the inhibition of cellular growth and transformation by IFN-α. An intriguing question is whether GAP activity is modulated in response to IFN-stimulated AA. In addition to modulation by tyrosine kinase (mitogenic) signaling pathways (*21*), GAP may be an early target of phospholipid hydrolysis mediated by non–tyrosine kinase (*22*) growth inhibitory receptors.

IFN-α receptor–mediated activation of ISRE-specific trans-acting factors provides a convenient experimental model for elucidating the precise role of PLA_2-generated lipid mediators in the coupling of environmental signals to specific changes in gene expression. Because of the large number of AA metabolites (*14, 16*) that could act as second messengers in signaling cascades, eicosanoid synthesis provides a mechanism for maintaining signal fidelity along the activation pathway between IFN-α receptor and cytoplasmic ISRE trans-acting factors. Our results suggest that stimulation of receptor-coupled PLA_2 and AA metabolism may participate in signaling cytokine-specific growth inhibitory responses through activation of latent transcription factors.

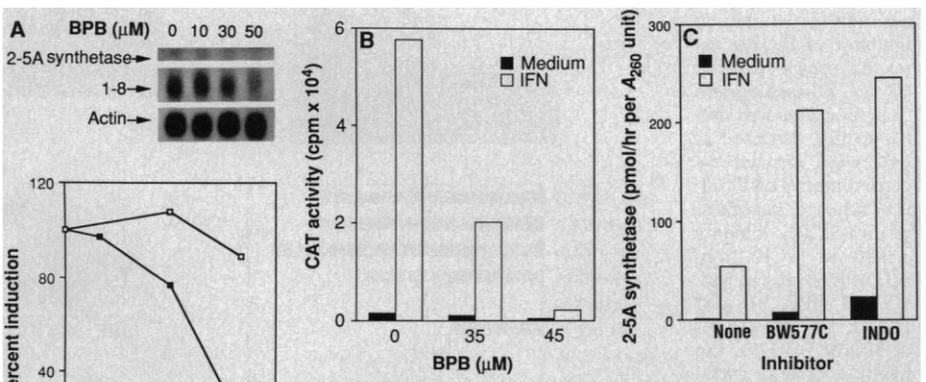

Fig. 3. Effect of inhibitors of AA metabolism on ISRE-dependent transcription activation and gene expression. (**A**) RNA from IFN-α–treated (2000 IU/ml) A31 cultures pretreated with the indicated concentrations of BPB was analyzed (*17*) for IFN-induced expression of the ISRE-containing genes 2-5A synthetase and 1-8, as well as a control actin gene. Relative densitometric quantitation of 1-8 and actin transcripts is indicated (the average of duplicate plates). (**B**) IFN-α–induced expression of a transfected ISRE-CAT gene (*17*) was analyzed in COS-1 cells that had been pretreated (15 min) with the indicated concentrations of phospholipase inhibitor BPB. (**C**) L929 fibroblasts were treated simultaneously with IFN-α/β and the lipoxygenase and cyclooxygenase inhibitors, BW577C (50 μM), or INDO (10 μM) and assayed for IFN-α induction of 2-5A synthetase activity (*17*) expressed as picomoles of [^{3}H]ATP polymerized per hour per absorbance unit (at 260 nm).

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5. Balb/c 3T3 fibroblasts (subclone A31) were obtained, at ~60 subculture passages, from the American Type Culture Collection. Confluent A31 monolayers (60-mm dishes) were labeled at 37°C for 2

hours with 100 μCi per dish of carrier-free [^{32}P]orthophosphate (Amersham), in minimal essential medium (MEM) that contained 0.5% young calf serum (YCS) and 100 mM Hepes buffer (pH 7.4). After ^{32}P-labeling, monolayers were washed three times with MEM with 0.5% YCS at 37°C, and treated in the same medium with or without recombinant human hybrid IFN-α [Δ4-HuIFN α_2/α_1, 2000 IU/ml (10 ng/ml), specific activity 2×10^8 IU/mg of protein, obtained from P. Trotta and T. Nagabushan], which is fully active on mouse cells. At indicated times the medium was aspirated, and monolayers were washed three times with Dulbecco's phosphate-buffered saline (Gibco) (PBS), 4°C. Ice-cold PBS (0.5 ml, pH 7.4) was then added to each dish, and cells were harvested by scraping. Cell suspensions were extracted and processed for analysis by TLC as described (*22*). Unlabeled standards for phosphatidylserine (PS), phosphatidylethanolamine (PE), PI, PC, lysoPE, lysoPI, and lysoPC (Sigma) were used to identify labeled species. ^{32}P-labeled lipids (15,000 cpm) were spotted onto a Silica Gel G TLC plate. Plates were developed as described (*23*). Autoradiography was performed with intensifying screens for 2 to 3 hours. Autoradiograms were scanned with an LKB Ultrascan densitometer, and integrated peaks representing each phospholipid species were expressed as a percentage of total labeled phospholipid. Similar results were obtained by scraping individual labeled species from the plates and counting radioactivity directly. In the experiment shown, unstimulated levels of lysoPC and PC were ~2% and ~77% of total ^{32}P-labeled lipid, respectively. For [^{3}H]AA release experiments, confluent A31 monolayers were labeled for 18 to 24 hours with [^{3}H]AA (0.2 mCi/ml, Du Pont Biotechnology Systems) in MEM with 0.5% YCS and 0.5% fetal calf serum. After labeling, monolayers were washed three times with MEM at 37°C, and MEM with IFNα (10 ng/ml) or PDGF (10 ng/ml, R&D Systems) was added at time 0. After 2 and 15 min at 37°C, aliquots from duplicate plates for each treatment were measured for [^{3}H]AA released to the culture medium. These results are representative of three independent experiments (n = 10). The mean [^{3}H]AA release for these experiments was 140 ± 16% (±SEM). [^{3}H]AA release was maximal between 1600 and 2000 IU of IFN-α per milliliter. In order to confirm uptake of label into appropriate pools, [^{3}H]AA-labeled phospholipids were analyzed by TLC (*23*). Typically, 30 to 35% of input [^{3}H]AA was incorporated into lipid. Under these conditions, the lipid profile of incorporated [^{3}H]AA was PC (50 to 52%), lysoPE (14 to 15%), lysoPC (9 to 10%), with the balance migrating equally as PE, PI, and PS species.

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11. Oligonucleotide binding sites were synthesized on an Applied Biosystems 380A DNA synthesizer. The sequences are as follows: ISRE, 5′-CCCTTCTC-GGGAAATGGAAACTGAAAATC; IRE, 5′-CAT-AGGAAAACTGAAAGGGAGAAGTGAAAGT-GGGAAATTCCTCTG; and *H-2*K^b, 5′-CGGCTG-GGGATTCCCCATCT. The ISRE represents nucleotides −80 to −52, relative to the presumed initiator AUG of the murine 2-5A synthetase gene (*2*). Underlined are the nucleotides comprising the inducible ISRE, as determined by contact point analyses in vitro, binding, and functional analysis of point mutations, and derived by sequence comparison of ISREs from a number of IFN-induced genes (*2*). The partial IRE encompasses nucleotides −99 to −55, relative to the transcription start site of the human IFN-β_1 gene (*13*). The H-2K^b oligonucleotide represents a sequence element located at position −165 of the *H-2*K^b gene of the murine major histocompatibility complex (*24*). These synthetic binding sites were end-labeled with T_4 DNA kinase in the presence of [γ-^{32}P]adenosine triphosphate ([γ-^{32}P]ATP), and purified on a Sephadex G-50 column. For EMSA, labeled oligonucleotides were incubated (20,000 to 30,000 cpm per reaction) with 5 μg of whole-cell extract (WCE) from confluent A31 monolayers. Whole-cell extracts were made as follows: cell pellets were resuspended in buffer A (low salt) (*25*) and incubated on ice for 5 min. Cells were then centrifuged for 10 s at ~10,000*g*. Pellets were then washed once in buffer A (*25*) and resuspended in buffer C (high salt) (*25*) for 10 min, on ice. Cell debris was pelleted, and crude extracts were clarified by centrifugation (18,000*g*) at 4°C for 30 min. Supernatants were dialyzed (4°C) against two changes (100 volumes) of buffer D (*25*) for a total of 5 hours. Aliquots of these extracts were stored at −70°C for use in EMSA reactions (*9*). All extracts were run two to four times on separate occasions to confirm relative binding activities. Autoradiography was for 19 to 30 hours with intensifying screens. Inhibitors were purchased from Sigma.

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17. Total cellular RNA was isolated and subjected to Northern analysis as described (*9*) with cDNA probes representing the murine 2-5A synthetase and 1-8 genes (*2*). Autoradiograms were scanned by densitometer for relative quantitation of RNA transcripts. Multiple film exposures ensured linear film response. After $CaPO_4$-mediated transfection of the ISRE-CAT hybrid gene (*9*), duplicate plates were treated with BPB at 35 or 45 μM in MEM, or with MEM alone. After a 15-min pretreatment IFN-α was added (2500 IU/ml), and cells were harvested at 10 hours. CAT activity was assayed as previously described (*9*). Under these conditions the variation in transfection efficiency was routinely within 10%, as assayed by β-galactosidase expression from transfected plasmids carrying the bacterial lacZ reporter gene. Duplicate plates within one experiment always agreed to within 10%. Each concentration of BPB was compared for induction by IFN-α against duplicate plates that received the drug only. For the 2-5A synthetase activity experiments, subconfluent L929 fibroblasts were treated with a subsaturating concentration of natural murine IFN-α/β (100 IU per 2×10^5 cells) for 20 hours. Cyclooxygenase and lipoxygenase inhibitors were included in the treatment period, either alone or with the IFN. INDO and BW577C treatments were as in the legend to Fig. 2B. After treatment, cells were harvested and assayed for 2-5A synthetase activity, as described (*9*).

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26. We wish to thank C. Lingwood for reagents and advice regarding the TLC experiments and F. Coceani for supplying BW577C, ETYA, AA, advice, and helpful discussions. AA861 was generously provided by Y. Oka. We also thank F. Coceani, T. Pawson, and C. Campbell for critical comments on the manuscript; G. Stark, I. Kerr, E. Rozengurt, A. Flenniken, and M. Rutherford for comments; R. Teteruk for photography; and M. Rutherford for supplying the IFN-β IRE and *H-2*K^b oligonucleotides. G.H. was the recipient of an Ontario Graduate Scholarship. Supported by a grant to B.W. from the National Cancer Institute (Canada).

12 July 1990; accepted 27 September 1990