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Coordinate Regulation of β-Lactamase Induction and Peptidoglycan Composition by the *amp* Operon

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The *amp* operon, which is located on the *Escherichia coli* chromosome, modulates the induction of plasmid-borne β-lactamase genes by extracellular β-lactam antibiotics. This suggests that the gene products AmpD and AmpE may function in the transduction of external signals. β-Lactam antibiotics are analogs of cell wall components that can be released during cell wall morphogenesis of enterobacteria. The *amp* operon was studied to determine its importance in signal transduction during cell wall morphogenesis. The peptidoglycan compositions of *amp* mutants were determined by high-performance liquid chromotography and fast atom bombardment mass spectrometry. When a chromosomal or plasmid-borne copy of *ampD* was present, the amount of pentapeptide-containing muropeptides in the cell wall increased upon addition of the cell wall constituent diaminopimelic acid to the growth medium. These results suggest that β-lactamase induction and modulation of the composition of the cell wall share elements of a regulatory circuit that involves AmpD. *Escherichia coli* requires AmpD to respond to extracellular signaling amino acids, such as diaminopimelic acid, and this signal transduction system may regulate peptidoglycan composition in response to cell wall turnover products.

THE HETEROPOLYMERIC PEPTIDOglycan of *Escherichia coli* is composed of over 40 different building blocks (*1*). The overall composition of this exoskeleton requires coordination of the activities of ten synthetic enzymes, which attach the *N*-acetylglucosaminyl-*N*-acetylmuramyl pentapeptide precursors to the preexisting sacculus, and at least nine cell wall hydrolases, which manicure new cell wall during maturation and turnover. The control mechanisms governing this complex system are unknown but may involve coordinate regulation with the synthesis of the other cellular macromolecules (*2*). In addition, efficient communication must exist between the intracellular site of muropeptide precursor synthesis and the extracellular site of cell wall assembly. For example, *E. coli* has an efficient system for uptake and reuse of cell wall peptides released during turnover and maturation (*3*). Also, β-lactam antibiotics probably do not enter the cytoplasm, yet they induce intracellular events such as activation of cell wall hydrolases (*4*) and expression of β-lactamases (*5*). Because β-lactam antibiotics are structurally analogous to the D-alanyl-D-alanine component of the cell wall stem peptide, we reasoned that the pathway that leads to induction of β-lactamase production might share regulatory components with the pathway for cell wall metabolism. Therefore, we sought to obtain mutants with aberrant cell wall metabolism by selecting mutants that were altered in β-lactamase induction.

Constitutive overproduction of β-lactamase is associated with mutations in the *amp* operon (*5*). The operon contains two chromosomal loci, *ampD*, which encodes a cytoplasmic protein, and *ampE*, which encodes a membrane-associated protein (*6, 7*). To test the hypothesis that mutants altered in the regulation of β-lactamase induction would also show differences in cell wall metabolism, we analyzed two series of mutants in the *amp* operon (*8–10*) for the composition of their cell walls. We introduced plasmids that carried *ampD*, *ampE*, or both genes into these mutant cells to determine the ability of these loci to complement the chromosomal *ampD* and *ampE* defects.

Differences in peptidoglycan composition were apparent between the parents and the *amp* operon mutants (Fig. 1 and Table 1) (*11, 12*). Two peptidoglycan components with retention times of 56 and 82 min, which accounted for 27 ± 4.1% (± standard errors) of the total wall composition of the wild-type strain, were reduced to a total of <0.5 ± 0.4% in the absence of the *amp* operon. We determined, by fast atom bombardment mass spectrometry (FAB MS), that the masses of the peaks were consistent with those of monomeric and dimeric pentapeptides, respectively (that is, *N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine and this pentapeptide monomer linked to an identical unit that was missing the terminal D-alanine). The loss of these pentapeptide-containing species in the deletion mutant was accompanied by a proportionate increase in the monomeric and dimeric tetrapeptides (increased from 54 to 76%), such that the relative total amounts of monomeric and dimeric muropeptides in the wild-type and mutant strains remained

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equivalent. Thus, the amount of pentapeptide in the cell wall was related to the *amp* genotype.

To determine if regulation of pentapeptide concentration was dependent on *ampD, ampE,* or both, we introduced the individual genes into the *amp* operon mutants, and we examined cell wall composition in these strains. Expression of functional AmpD, confirmed by minicell analysis, increased the amounts of monomeric and dimeric pentapeptides in the mutants to 44 ± 3.5 and 71 ± 6.2%, respectively, of wild-type values (Table 1). No changes were observed with a functional *ampE* gene or a nonfunctional *ampD* gene.

We examined the possibility that the amount of pentapeptide in wild-type strains was determined by a signal in the medium that depended on an intact *amp* operon. To identify signaling molecules, we determined variation of peptidoglycan composition as a function of the presence of exogenous peptidoglycan-derived amino acids or peptides. High concentrations of pentapeptide-containing muropeptides were observed only when strains that expressed AmpD were grown in the presence of diaminopimelic acid. Addition of other cell wall components (D-alanine, D-alanyl-D-alanine) had no effect on pentapeptide concentration. These results suggest that *ampD* participates in the regulation of cell wall metabolism by diaminopimelic acid.

To analyze the mechanisms by which AmpD generated changes in peptidoglycan composition, we examined the effects of mutations in *ampD* on the synthetic and degradative arms of peptidoglycan metabolism. No differences in the molecular sizes, the amounts, or the number of cell wall synthetic enzymes were observed in the mutants (Fig. 2). Thus, the preservation of intact pentapeptides in the cell wall probably did not result from alteration of the enzymes that incorporate precursors into the cell wall. Next, cell wall hydrolase activities were measured in cytoplasmic and membrane extracts of the *ampD* mutants. The activities of the soluble and membrane-bound forms of the degradative transglycosylase and endopeptidase did not differ in wild-type or mutant extracts (Table 2).

Table 2. Degradative transglycosylase and endopeptidase activities in *amp* mutants. Extracts from cultures grown in the presence of diaminopimelic acid of wild-type (strain SN03) and *ampD*, *ampE* mutant (SN0302) and the same strains harboring *ampD* on plasmid pNU405 were analyzed for soluble and membrane-bound endopeptidase and transglycosylase activities in cell extracts at equivalent protein concentrations (*18–20*). Numbers given are arbitrary units with 100 being equal to the specific activity of the transglycosylase (endopeptidase) in crude extracts from wild-type cells. All triplicate values were within 11% of the mean.

Phenotype	Transglycosylase activity		Endopeptidase activity	
	Soluble	Membrane-bound	Soluble	Membrane-bound
Wild-type	100	100	100	100
Wild-type + *ampD*	94.9	94.5	88.2	105.3
Mutant	96.7	94.3	119	98.9
Mutant + *ampD*	97.0	94.8	79.5	99.2

Carboxypeptidases cleave pentapeptides to yield tripeptides and tetrapeptides. Therefore, the decrease in the concentration of the pentapeptide in the mutant cell wall may have resulted from an increase in carboxypeptidase activity. As an indicator of carboxypeptidase activity on intact cell wall (most cellular carboxypeptidases hydrolyze only released material), we analyzed the mutant and wild-type cell walls for tripeptides and tetrapeptides, the products of this enzymatic reaction. Wild-type cell walls contained 58.5 ± 1.7% total tripeptides and tetrapeptides whereas the mutant contained 68.4 ± 0.9% ($P < 0.01$, Neuman-Keuls test). Products of the carboxypeptidase reaction were reduced to wild-type amounts by introduction of *ampD* (60.7 ± 1.2%, $P > 0.5$ compared to wild type), but not *ampE* (68.1 ± 1.5%, $P < 0.01$ compared to wild type). These results are consistent with the hypothesis that ampD represses modification of the cell wall by carboxypeptidases.

A decrease in carboxypeptidase activity in strains that express AmpD should not change the rate of cell wall turnover (as determined by loss of carbohydrate from the wall) but should decrease release of peptide

Table 1. Peptidoglycan composition of wild-type and mutant strains of *E. coli*. Strains were grown in the presence of diaminopimelic acid, and cell wall was prepared for HPLC analysis. The wild-type (HfrH, D+E+) pattern was compared to those of the double mutant strain (JRG582, D−E−) and its derivatives carrying *ampE* (pNU435, D−E+), *ampD* (pNU436, D+E−), or inactive *ampD* and *ampE* (pNU437, D−E−$_i$). Muropeptides are designated by the length of the peptide side chain attached to the *N*-acetylglucosaminyl-*N*-acetylmuramyl moiety: Tri = L-alanyl-D-glutamyl-*meso*-diaminopimelic acid, Tetra = Tri + D-alanine, Penta = Tetra + D-alanine. More than one peptide designation indicates a dimeric bisdisaccharide form attached by a diaminopimelyl-D-alanine crossbridge or a diaminopimelyl-diaminopimelic acid (Dap2) crossbridge. Substitutions to the muropeptide: Lys-Arg = lysyl-arginyl residue of lipoprotein, Anh = 1,6 anhydromuramic acid. Values are means of triplicate experiments with a maximum SEM of 2.6%.

Muropeptide	% of total peptidoglycan				
	D+E+	D−E−	D−E+	D+E−	D−E−$_i$
Tri	10.7	10.6	6.6	10.2	7.7
Tetra	40.7	45.8	53.0	33.9	47.9
Penta	14.6	0.0	0.0	6.5	0.10
Tri-LysArg	2.4	3.2	2.2	4.4	1.2
Tri-Tri-Dap2	0.4	0.4	0.1	1.8	0.2
Tetra-Tri-Dap2	3.1	3.7	3.3	3.0	2.7
Tri-Anh	1.2	4.7	2.9	7.4	3.5
Tetra-Tetra	13.6	31.1	31.2	23.4	30.6
Tetra-Penta	13.2	0.4	0.7	9.4	0.4

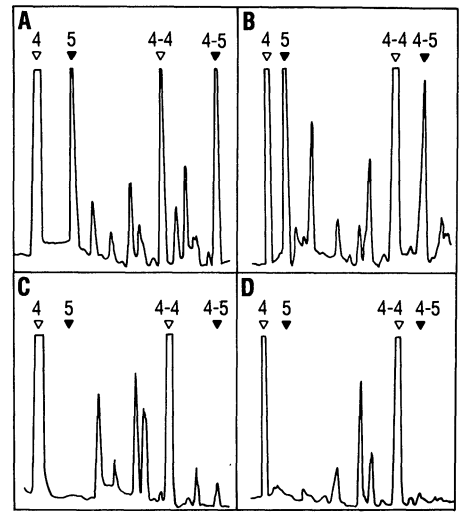

Fig. 1. HPLC pattern of peptidoglycan from strains that differed in expression of the *amp* operon. (**A**) The wild-type strain (HfrH) and the *ampD, ampE* deletion mutant (JRG582) were analyzed carrying (**B**) the plasmid with insertion of *ampD* (JRG582pNU436), (**C**) the plasmid backbone (JRG582pACYC184), or (**D**) the plasmid with insertion of *ampE* (JRG582pNU435). Positions marked with closed arrowheads change with the phenotype; positions marked with open arrowheads indicate the constant major monomeric and dimeric component of the peptidoglycan. (4 = monomeric disaccharide tetrapeptide; 5 = monomeric disaccharide pentapeptide; 4-4 = dimeric bisdisaccharide tetra-tetrapeptide; and 4-5 = dimeric bisdisaccharide tetra-pentapeptide.)

Table 3. Effects of *ampD* and *ampE* on the release of cell wall peptides. Bacteria were grown overnight in [^{3}H]diaminopimelic acid (1 mCi/ml, New England Nuclear). Triplicate cultures were filtered and diluted, and duplicate samples (200 μl) were taken at 0, 2, 4, 6, and 24 hours. The amount of radioactivity remaining in material that was precipited by boiling in SDS (5%) was determined as a measure of labeled components remaining in the wall (*3*). Values are mean ± SD of the percent of [^{3}H] counts per minute released from the cell wall per bacterial generation time of 30 min. Strains are as described in (*10*).

Strain	Chromosomal phenotype	Plasmid phenotype	% release of cell wall peptides per 30 min
SN03	D+E+		3.6 ± 0.6
SN0301	D−E+		6.5 ± 0.4*
		D+E−	1.2 ± 0.4
SN0302	D−E−		7.0 ± 2.3*
		D+E−	2.3 ± 1.1
		D+E+	0.6 ± 2.7
HfrH	D+E+	D−E−	2.2 ± 1.2
JRG582	$D-E-_i$	D−E−	6.2 ± 1.4*
		D−E+	3.5 ± 0.3
		D+E−	1.7 ± 0.6
		D−E−	5.7 ± 0.8*
		D+E+	1.2 ± 0.2

*$P < 0.01$, as compared to SN03 (Neuman-Keuls test).

subcomponents (as determined by loss of peptide from the wall). As predicted, all strains, regardless of *amp* genotype, had a similar rate of loss of carbohydrate ([^{3}H]*N*-acetylglucosamine) from the cell wall (45 to 56 ± 9% per generation). However, metabolism of cell wall peptides differed in *ampD*, but not *ampE*, mutants (Table 3). The *ampD* mutants released approximately twice the amount of [^{3}H]diaminopimelic acid into the medium as the wild-type strain. The defect was complemented by introduction of *ampD* on a plasmid.

In this report, we have shown that, in wild-type *E. coli*, *ampD* is required for response to extracellular diaminopimelic acid, and that *ampD* contributes to an increase in pentapeptides in the cell wall. We propose that the *ampD* gene product represses a carboxypeptidase that hydrolyzes the cell wall pentapeptide. The *ampD* gene product represses the expression of β-lactamase in the absence of β-lactam inducer. These studies indicate that *ampD* coordinately regulates both β-lactamase expression and peptidoglycan metabolism, and that mutants altered in peptidoglycan composition can be selected by screening for defects in the regulation of β-lactamase production. It is reasonable that these two phenomena could share elements of regulation, because β-lactam antibiotics are analogs of the pentapeptide and β-lactamase may be evolutionarily related to carboxypeptidases (*13*). Mutations in *ampD* that enhance β-lactamase production constitute the most common mechanism by which enterobacteria become antibiotic-resistant (*5*). Changes in cell wall composition that arise from mutation of *ampD* do not appear to adversely affect the physiology of the bacteria. Thus, an understanding of the function of the *amp* operon is of considerable therapeutic interest.

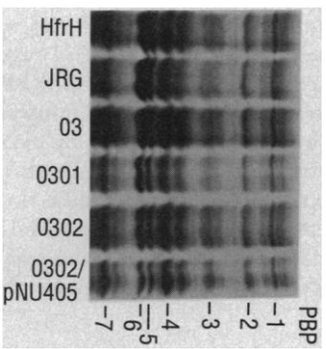

Fig. 2. Effect of mutation of the *amp* operon on the cell wall synthetic enzyme profile. Cell wall synthetic enzymes were detected by covalent binding of [^{3}H]benzylpenicillin (10 μg or 600 μCi/ml, Merck Research Division). The PBP pattern for each strain was determined in whole cells at equivalent cell densities (*16*) and in membrane preparations at equivalent protein concentrations (*17*). Amounts of individual PBP's, quantitated by scanning densitometry, were not different between lanes (range of percent of total band density per lane): band 1 = 9 to 12%; band 2 = 8 to 11%; band 3 = 2 to 4%; band 4 = 32 to 39%; bands 5 and 6 complex = 36 to 41%; band 7 = 12 to 15%.

The nature of the link between β-lactamase induction and cell wall metabolism is unknown. Genetic inactivation of penicillin-binding protein (PBP)1A, 1B, 2, or 3 (ts mutation) did not abolish inducibility, suggesting that none of the high molecular size synthetic transpeptidase-transglycosylases are required for β-lactamase induction (*14*). No mutants totally defective in carboxypeptidase activity have been isolated. However, strains with an increased amount of PBP4-related carboxypeptidase become hyperinducible by β-lactams relative to the wild type (*14, 15*). This finding reinforces the link between carboxypeptidases and β-lactamase induction proposed here. It is possible that a low molecular size ligand that is generated during cell wall metabolism enters the cell during recycling of peptide components. Support for this model comes from the observations that diaminopimelic acid can induce β-lactamase production (*15*) and can alter cell wall composition in *ampD*-containing bacteria.

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10. SN0301 (*ampD1*) and SN0302 (*ampD2*) are spontaneous mutants derived from SN03 (*ampA1, ampC8, pryB, recA, rpsL*) (*5*). JRG582 Δ(*nadC-aroP*)2, a derivative of HfrH, is totally deleted for the *amp* operon (*8*). Plasmids pNU435, pNU436, and pNU437 are derivatives of pNU401 (*5*). Plasmid pNU401 carries the *ampD*, *ampE* region of *E. coli* on a 6.1-kb Bam HI–Hind III fragment cloned into pACYC184 (*9*). To create pNU435, a Bgl II–linker (Pharmacia) was inserted into the Sph I site in the *ampD* gene resulting in an out-of-frame mutation in AmpD after 95 amino acids. Plasmids pNU436 and pNU437 are Ava I cutbacks of pNU401 and pNU435, respectively. The 2.8-kb deletion in pNU436 and pNU437 starts within the *ampE* gene resulting in expression of a 40–amino acid truncated form of AmpE from these constructs.
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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.).

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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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