Cooperative Role of Interferon Regulatory Factor 1 and p91 (STAT1) Response Elements in Interferon-γ-inducible Expression of Human Indoleamine 2,3-Dioxygenase Gene*

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Interferon (IFN)-γ induces the expression of the indoleamine 2,3-dioxygenase (INDO) gene in human cells, which plays a role in the inhibitory effect of IFN-γ on intracellular pathogens and on cell proliferation. Earlier studies established that the IFN-γ-inducible expression of the INDO gene was dependent on two upstream elements: (i) a 14-base pair sequence homologous to an interferon-stimulated response element (ISRE) sequence found in IFN-α-inducible genes and (ii) a 9-base pair palindromic sequence (palindromic element (PE) II) homologous to an interferon-γ-activated site (GAS) element found in IFN-γ-inducible genes. A second GAS element (PE I), between ISRE and PE II, was ineffective in supporting a response to IFN-γ. Studies were carried out to determine the distinction between the two GAS elements and the relative role of the two elements (ISRE and PE II) required for a response to IFN-γ. The PE I element was able to form a complex with IFN-γ-activated p91 (STAT1) factor but with lower efficiency than the complex formed with PE II sequence. However, switching the positions of PE I and II sequences in reporter plasmid constructs (containing chloramphenicol acetyltransferase gene) showed that both PE I and PE II were able to support a response to IFN-γ if located at the position of PE II but not at the position of PE I. Increasing the distance between the ISRE and PE II also affected the level of response, suggesting that the relative position of the two elements is important for optimal stimulus. To explore whether an interaction between the IFN-γ-regulated factors (IRF-1 and p91) binding to the ISRE and PE II might be important, we tested whether the ISRE sequence could be replaced by another response element, NF-κB. The plasmid construct with NF-κB element in place of the ISRE was responsive to IFN-γ, indicating that an interaction between the IRF-1 and p91 factors was not required. The results indicate that the response of INDO gene to IFN-γ depends on a cooperative role of IFN-γ-responsive factors binding to the ISRE and GAS elements.

Interferon (IFN)-γ, a pleiotropic cytokine produced primar-
spect, IFN-γ-inducible expression of INDO gene differs from a number of other cellular genes containing GAS sequences that are induced by IFN-γ without the need for an ISRE sequence or IFN-γ-induced IRF-1 factor, for example the GBP (19), FcγR1 (20), Ly-6E (21), and mlg (22) genes. Furthermore, the IFN-γ-responsive sequence from the INDO gene actually contains two GAS-related sequences (PE I and PE II), but mutagenesis of the PE II sequence (leaving PE I intact) abolished the response to IFN-γ, indicating that the PE I sequence was inadequate in supporting IFN-γ inducibility (15). Alternatively, since two elements (ISRE and PE II) were required for IFN-γ response, it is possible that a proper distance or alignment of the two elements may be important for an optimal cooperation and that PE I is out of proper alignment. Such a situation may suggest the possibility of an interaction between the two sequence elements through their binding factors (IRF-1 and p91). Therefore, we have carried out further studies on the relationship between the two control elements in the IFN-γ-responsive sequence from the INDO gene in the IFN-γ-inducible expression of plasmid constructs.

MATERIALS AND METHODS

GM00637 (SV40-transformed human fibroblasts) obtained from the Coriell Institute for Medical Research (Camden, NJ) were grown in Eagle's minimal essential medium supplemented with 5% bovine serum, 5% newborn bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Purified recombinant IFN-γ (specific activity of 2 × 10⁷ National Institutes of Health reference units/mg of protein) was a generous gift from Genentech, Inc. (South San Francisco, CA). Synthetic oligodeoxynucleotides were obtained from DNA International, Inc. (Lake Oswego, OR) or Oligos, Etc., Inc. (Guilford, CT).

DNA Plasmids—Earlier studies have identified two sequence elements (ISRE and PE I) in a 48-bp region (−449 to −402) upstream of the INDO gene that determine the response to IFN-γ (15). Reporter plasmids with chloramphenicol acetyltransferase (CAT) gene were constructed in which this sequence (−451 to −402) or its variants were cloned 5′ of the TK-CAT gene in plj CAT2 phagemid vector (Fig. 1D). This 50-bp sequence (−451 to −402) was constructed by using two synthetic oligonucleotides representing 5′-halves of the upper and lower strands with 11-bp overlaps and SphI and SalI restricting sites added to the two termini (see Fig. 1B). The two oligos were annealed and then extended by incubation with Klenow enzyme and all four dNTPs (Fig. 1B). The reaction mixtures were then heated to 75°C for 10 min and allowed to cool slowly, and the oligos were precipitated with ethanol. The product was digested with SphI and SalI restriction enzymes, and the fragment was ligated to plj CAT2 vector (containing herpesvirus thymidine kinase promoter) that had been digested with SphI and SalI enzymes. Competent Escherichia coli DH1 cells were transformed with the construct, and plasmids were isolated from several colonies and analyzed for the presence of the cloned insert by restriction analysis.

Several variants of this 50-bp fragment were constructed in which the sequence of the ISRE or the PE I or II was modified (Fig. 1C). This was achieved by using variants of the upper or lower oligonucleotide with the desired sequence modification. The oligos were annealed, extended, and then cloned in plj CAT2 vector as outlined above for the wild-type construct. The sequence of the cloned 50-bp fragments in different constructs was verified by sequence analysis (25).

Transfection Assays—The various plasmid constructs were tested for their response to IFN-γ in transient transfection assays using GM00637 cells as described earlier (15). In all transfections, plasmid RSVZ (containing bacterial β-galactosidase gene) was included as an internal control. In all experiments, cells were transfected in each 100-mm plate divided into two 60-mm plates where one was used as a control and the other for treatment with IFN-γ, thereby minimizing variability due to possible differences in the transfection efficiency in different cultures. The amounts of cell lysates for CAT assays were normalized according to the β-galactosidase activities. Each experiment was repeated at least three times.

Preparation of Cell Extracts and Protein-DNA Binding Assays—GM0637 cells were grown in 175-cm² flasks, and one group was treated with IFN-γ (400 units/ml) for 15 min and the other group served as control. Whole cell extracts were prepared by a modification of the described procedure (26). In brief, cells were washed three times with cold phosphate-buffered saline, harvested in phosphate-buffered saline with rubber scrapers, and pelleted by centrifugation (500 × g, 10 min). The cell pellets were suspended in 1–1.5 pellet volumes of buffer C (20 mM HEPES/KOH, pH 7.9, 20% glycerol, 0.42 mM NaN₃, 1.5 mM MgCl₂, 0.2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 0.5 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 10 μg/ml pepstatin). The resulting suspension was stirred gently with a magnetic stirring bar for 30 min at 4°C and centrifuged at 100,000 × g for 30 min, and the resulting supernatants were dialyzed against buffer D (20 mM HEPES/KOH, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM sodium orthovanadate) for at least 5 h. The dialysates were clarified by centrifugation at 16,000 × g for 20 min, quick frozen in aliquots in liquid nitrogen, and stored at −80°C. Protein concentration was assayed by the Bradford procedure (27) using protein assay reagent from Bio-Rad and bovine serum albumin as standard.

Whole cell extracts were tested for IFN-γ-regulated DNA binding factors, p91 and IRF-1, in electrophoretic mobility shift assays. The probes used were (i) a 13-bp oligonucleotide containing the PE I sequence (–425 to –413) and (ii) a 13-bp oligonucleotide (–412 to –400) containing the PE II sequence found in the INDO gene promoter.

![Fig. 1. A, nucleotide sequence of the IFN-γ-responsive 50-bp region (–451 to –402) of the INDO gene promoter; B and C, construction of a –451 to –402 subfragment and its variants (with added restriction sites) for cloning in plj CAT2 vector 5′ of the TK-CAT gene; D, map of plj CAT2 vector that was derived from plj CAT1 vector (23) by the insertion of herpes simplex virus TK promoter isolated from pBLCAT2 vector (24). The plj CAT2 vector was kindly provided by Dr. J. Fridovich-Keil.](image-url)
indicated that this IFN-γ-induced complex was formed with the PE I sequence. Antibody to p91 factor yielded a supershifted complex (lane 9) that was not obtained with non-immune IgG (lane 8). Thus, the PE I sequence was able to form a complex with p91 factor. However, a comparison of the PE I and II oligomer probes in this assay indicated that the PE II probe gave a stronger signal than the PE I probe (Fig. 2A, lane 2). The complex obtained with the PE II probe was abolished by unlabeled PE II oligomer but not by the ISRE oligomer, and it was supershifted by antibody to p91 (Fig. 2A). A comparison of unlabeled PE I and II oligomers that were added for competition showed that whereas the PE II oligomer completely abolished the complex formation when added at 100-fold molar excess, the PE I oligomer competed only partially (Fig. 2B), indicating that the PE II oligomer was much more efficient in competing for this factor than the PE I oligomer. This is further illustrated in Fig. 2C in which increasing amounts of unlabeled PE I or II oligomers were added for competition against p91 binding to labeled PE II probe. The results showed that PE II oligomer efficiently competed when added at 10- or 20-fold molar excess and essentially abolished the complex formation when added at 50-fold excess. In contrast, the PE I oligomer showed only partial competition when added at 100-fold molar excess and only a marginal reduction at 20- or 50-fold excess. Thus, the PE I sequence was less efficient than PE II sequence in forming a complex with p91 factor. Whether the inefficiency of the PE I sequence to bind the p91 factor could account for its inability to support a response to IFN-γ (when PE II had been mutated) or whether its location in relation to the ISRE sequence was a contributing factor was examined by making constructs in which the positions of the PE I and II were switched and the other GAS-related sequence was replaced by a mutated sequence as illustrated in Fig. 1C.

As mentioned above, the IFN-γ-responsive region of the human INDO gene identified earlier contains two GAS-related sequences, PE I and PE II, but whereas PE II was effective in supporting a response to IFN-γ in the presence of the ISRE sequence, PE I was not (15). To determine whether the PE I sequence was inadequate for IFN-γ response or that a proper distance or alignment with the ISRE sequence might be important, we tested whether the PE I sequence was able to form a complex with the IFN-γ-activated p91 (STAT1) factor and, if so, whether the location of the GAS-related sequence is important in relation to the ISRE sequence for a response to IFN-γ.

Fig. 2A shows an experiment in which a synthetic DNA oligomer containing the PE I and flanking sequence was used as a probe to test whether it would form a complex with IFN-γ-activated p91 factor. The results show that a complex with IFN-γ-regulated factor was indeed obtained (lane 2). The formation of this complex was competed out by oligonucleotides containing the PE I or II sequences (lanes 3 and 4) as well as by an oligomer from Ly-6E gene containing the GAS-related sequence (lane 5) but not by a mutated PE II oligomer (lane 6). An oligomer containing the ISRE and flanking sequence from the INDO gene was also unable to compete (lane 7). These results indicated that this IFN-γ-induced complex was formed with the PE I sequence. Antibody to p91 factor yielded a supershifted complex (lane 9) that was not obtained with non-immune IgG (lane 8). Thus, the PE I sequence was able to form a complex with p91 factor. However, a comparison of the PE I and II oligomer probes in this assay indicated that the PE II probe gave a stronger signal than the PE I probe (Fig. 2B, lane 2). The complex obtained with the PE II probe was abolished by unlabeled PE II oligomer but not by the ISRE oligomer, and it was supershifted by antibody to p91 (Fig. 2B). A comparison of unlabeled PE I and II oligomers that were added for competition showed that whereas the PE II oligomer completely abolished the complex formation when added at 100-fold molar excess, the PE I oligomer competed only partially (Fig. 2B), indicating that the PE II oligomer was much more efficient in competing for this factor than the PE I oligomer. This is further illustrated in Fig. 2C in which increasing amounts of unlabeled PE I or II oligomers were added for competition against p91 binding to labeled PE II probe. The results showed that PE II oligomer efficiently competed when added at 10- or 20-fold molar excess and essentially abolished the complex formation when added at 50-fold excess. In contrast, the PE I oligomer showed only partial competition when added at 100-fold molar excess and only a marginal reduction at 20- or 50-fold excess. Thus, the PE I sequence was less efficient than PE II sequence in forming a complex with p91 factor.

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**RESULTS**

As mentioned above, the IFN-γ-responsive region of the human INDO gene identified earlier contains two GAS-related sequences, PE I and PE II, but whereas PE II was effective in supporting a response to IFN-γ in the presence of the ISRE sequence, PE I was not (15). To determine whether the PE I sequence was inadequate for IFN-γ response or that a proper distance or alignment with the ISRE sequence might be important, we tested whether the PE I sequence was able to form a complex with the IFN-γ-activated p91 (STAT1) factor and, if so, whether the location of the GAS-related sequence is important in relation to the ISRE sequence for a response to IFN-γ.

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Whether the inefficiency of the PE I sequence to bind the p91 factor could account for its inability to support a response to IFN-γ (when PE II had been mutated) or whether its location in relation to the ISRE sequence was a contributing factor was examined by making constructs in which the positions of the PE I and II were switched and the other GAS-related sequence was replaced by a mutated sequence as illustrated in Fig. 1C.
of the PE II sequence abolished its capacity to support a response to IFN-γ, whether introduced in either the first three or last three bases (Fig. 3).

These results with a CAT reporter gene construct showed that expression of CAT activity from plasmid constructs and either treated with IFN-γ or not treated (C) or treated with IFN-γ alone (300 units/ml γ), or PMA alone (10 ng/ml or 100 ng/ml), or both together for 24 h. Cell extracts were prepared and assayed for CAT activity. The percent conversion of [14C]chloramphenicol to acetylated products was quantitated as described in Fig. 3.

The results suggest that the relative position of the GAS-related sequence is important, perhaps in relation to the ISRE sequence. To test this possibility further, we made constructs in which the spacing between the ISRE and PE II sequences was increased by inserting an additional 5 or 10 bp in plasmid A (with mutated PE I sequence), as illustrated in Fig. 1C (construct D). These constructs showed a greatly reduced induction of CAT expression in response to IFN-γ as compared with the parent plasmid (Fig. 3B). Therefore, either reducing the distance between the ISRE and PE II (Fig. 3A, construct B) or increasing this distance (Fig. 3B, constructs D5 and D10) markedly diminished the response to IFN-γ in transfected cells transfection assays, suggesting that the spacing between the ISRE and PE II is important for an optimal response to IFN-γ.

A requirement for two elements (ISRE and PE II/GAS) and a proper spacing between them suggested the possibility that their IFN-γ-regulated binding factors (IRF-1 and p91) might interact with each other thereby contributing to the IFN-γ inducibility. Alternatively, the two elements and their binding factors may be weak inducers individually but allow a strong induction cooperatively when present together in an optimal arrangement. To distinguish between these two possibilities, we asked whether the ISRE sequence could be replaced by another response element. We made a construct in which the ISRE sequence was replaced by an NF-κB element. The response of such a construct to IFN-γ or phorbol ester (PMA), or both, was tested. The expression of CAT activity from this construct was stimulated by IFN-γ and further boosted by PMA plus IFN-γ, but PMA alone showed little or no effect (Fig. 4). Therefore, the ISRE element could be effectively replaced by another response element, NF-κB, that could cooperate for IFN-γ-induced expression although it was not as effective as the ISRE element. This result indicates that the requirement for ISRE/IRF-1 was not absolute and that it could be replaced by another positive response element.

To determine whether the response of the construct E to IFN-γ involved an NF-κB factor (28) or whether the modified position of PE I, was ineffective in supporting a response to IFN-γ. Construct A, which contained the PE II sequence in the original location and a mutated PE I sequence, showed a normal response to IFN-γ, as shown earlier (15). Therefore, either the PE I or PE II sequence was able to support a response to IFN-γ if located at the position of PE II but not if located at the position of PE I.

Fig. 3. A, the effect of switching the position of GAS-related sequences (PE I and II) in the INDO gene promoter on the response of CAT reporter gene constructs to IFN-γ. Plasmids containing either the wild-type 50-bp IFN-γ-responsive sequence from the INDO gene promoter (50WT, see Fig. 1A) or its variant in which the ISRE sequence was replaced by NF-κB sequence (GGGGACTTTCC, construct E, see Fig. 1C) cloned 5′ to the TK promoter in pcCAT2 vector were tested for their response to IFN-γ in transient transfaction assays. The figure shows CAT activity in extracts of cells transfected with different plasmid constructs and either treated with IFN-γ (300 units/ml for 24 h) (γ) or not treated (C). The percent conversion of [14C]chloramphenicol to acetylated products was quantitated as described in Fig. 3. B, the effect of inserting an additional 5′ or 10 bp next to PE II sequence in the original location and a mutated PE I sequence, showed a markedly diminished response to IFN-γ plus IFN-γ, but PMA alone showed little or no effect (Fig. 4). Therefore, either reducing the distance between the ISRE and PE II (Fig. 3A, construct B) or increasing this distance (Fig. 3B, constructs D5 and D10) markedly diminished the response to IFN-γ, suggesting that the spacing between the ISRE and PE II is important for an optimal response to IFN-γ.

For instance, in plasmid construct C, the PE II sequence was replaced by PE I, and the original PE I sequence was mutated. Such a construct was used to ask whether the PE II sequence, which was ineffective in its original location, would be functionally active in supporting response to IFN-γ when located in the position of the PE II sequence. Experiments showed that expression of CAT activity from plasmid construct C was induced by IFN-γ to an extent similar to that of the wild type (Fig. 3A). This result indicates that PE I was able to functionally replace PE II and suggests that the original location of PE I may be inadequate in supporting response to IFN-γ. This was further supported by experiments with a reciprocal construct in which the PE I was replaced by the PE II sequence and the original PE II sequence was mutated (Fig. 1C, construct B). It was demonstrated earlier that the sequence changes introduced in either the first three or last three bases of the PE II sequence abolished its capacity to support a response to IFN-γ (15). Construct B showed no response to IFN-γ (Fig. 3A), indicating that the PE II element, when located in the

| Plasmid | 50WT | A | D5 | D10 |
|---------|------|---|----|-----|
| Percent Conversion | 0.5 | 22.2 | 0.9 | 27.4 | 1.1 | 30.4 | 0.3 | 0.3 |

| Plasmid | 50WT | A | E | E + PMA 10 | E + PMA 100 |
|---------|------|---|---|------------|-------------|
| Percent Conversion | 0.9 | 33.7 | 1.2 | 8.7 | 1.3 | 19.0 | 1.0 | 11.0 |

FIG. 4. Replacement of the ISRE element by NF-κB element in the IFN-γ-responsive sequence of the INDO gene. Plasmids containing either the wild-type 50-bp IFN-γ-responsive sequence (50WT, see Fig. 1A) or its variant in which the ISRE sequence was replaced by NF-κB sequence (GGGGACTTTCC, construct E, see Fig. 1C) cloned 5′ to the TK promoter in pcCAT2 vector were tested for their response to IFN-γ or PMA, or both, in transient transfaction assays. The cells were transfected with the two plasmid constructs and either not treated (C) or treated with IFN-γ alone (300 units/ml γ), or PMA alone (10 ng/ml or 100 ng/ml), or both together for 24 h. Cell extracts were prepared and assayed for CAT activity. The percent conversion of [14C]chloramphenicol to acetylated products was quantitated as described in Fig. 3.
sequence was still able to bind IRF-1 factor to some cryptic sequence element, the NF-κB and flanking sequence from construct E were used as a probe to test whether it would bind NF-κB and/or IRF-1 factor. The corresponding wild-type sequence was used in parallel for comparison. The results showed that (a) the NF-κB-modified probe did not yield a complex with the IRF-1 factor as obtained with the original sequence containing ISRE (Fig. 5, lane 3 versus lane 9), and the NF-κB oligomer when added at 100-fold molar excess did not compete for IRF-1 binding to the ISRE probe (lane 5), whereas the ISRE oligomer did (lane 4); and (b) the NF-κB probe yielded a different complex induced by IFN-γ (lane 9). The formation of this complex was competed out by the unlabeled NF-κB oligomer (lane 11) but not by the ISRE oligomer (lane 10), and it was supershifted by antibody to the NF-κB p65 component (lane 13) but not by non-immune Ig. A similar complex was obtained when cells were treated with PMA (10 or 100 ng/ml), which is known to activate NF-κB (29) (data not shown). These results indicate that this complex was formed with the NF-κB factor, which was induced by IFN-γ, and that the NF-κB probe did not bind the IRF-1 factor. The induction of the NF-κB factor by IFN-γ, together with p91 (STAT1), may account for the response of plasmid construct E to IFN-γ (Fig. 4). The NF-κB factor was induced more strongly by PMA than by IFN-γ (data not shown), which may explain a stronger induction when PMA and IFN-γ were added together (Fig. 4). However, PMA alone did not induce the expression of plasmid construct E, suggesting that activation of NF-κB alone was insufficient.

**DISCUSSION**

Our earlier results indicating that the response of the INDO gene promoter region to IFN-γ was dependent on two regulatory elements (ISRE and PE II/GAS) prompted us to study the relationship between these two elements. The fact that a second GAS-related sequence (PE I) was ineffective in supporting a response to IFN-γ in the original position but could do so when placed in the position of PE II suggested that the relative position of the two elements may be important. This was supported by the greatly diminished response of constructs in which the distance between the ISRE and PE II was either reduced when PE II was inserted in place of PE I (construct B in Fig. 3A) or increased by the insertion of an additional 5- or 10-bp sequence (constructs D5 and D10 in Fig. 3B). These results raised the possibility that the IFN-γ-regulated protein factors binding to these two sequence elements (IRF-1 and p91) might interact with each other for an optimal response. However, the fact that construct D with an additional 10-bp insertion between the ISRE and PE II also showed greatly reduced response to IFN-γ does not favor this possibility since a 10-bp insertion will add one full turn to the DNA helix and, therefore, retain the relative orientation of the two elements. Furthermore, the fact that the ISRE sequence could be replaced by another positive response element, NF-κB, argues against a dependence on a specific interaction between p91 and IRF-1 for the induction by IFN-γ. Therefore, the results indicate a cooperative role of IRF-1 and p91 (STAT1) in the induction of the INDO gene by IFN-γ.

It should be pointed out that a dependence on the IRF-1 factor has also been reported for the response of the inducible nitric oxide synthase (iNOS) gene to IFN-γ and lipopolysaccharide in mouse macrophages. Macrophages from IRF-1 knockout mice showed little or no induction of iNOS expression in response to IFN-γ and lipopolysaccharide (30). The iNOS gene promoter region contains an IRF-1 response element with homology to ISRE as well as GAS-related sequence elements in the vicinity (31, 32). Involvement of IRF-1 may explain the dependence on new protein synthesis for IFN-γ-inducible expression of the iNOS (33) and INDO genes (18).

The IFN-γ-inducible expression of FcγRI gene represents another example of a cooperative role of an accessory element for an optimal response to IFN-γ. The IFN-γ-responsive region of FcγRI gene contains a GAS sequence, but an additional (unidentified) promoter element is needed for an optimal response to IFN-γ (20). In view of our studies with the INDO gene and other examples cited above, it appears that whereas the GAS element may be a primary target for gene regulation by IFN-γ, the actual response of several genes may depend on a cooperative role of additional accessory elements in the promoter region.

The plasmid construct E in which the ISRE sequence was replaced with an NF-κB binding sequence showed induced expression in the presence of IFN-γ. Evidence was obtained, indicating that treatment with IFN-γ resulted in the induction of NF-κB activity (Fig. 5). However, induction of NF-κB by treatment with PMA was insufficient to induce the expression of this plasmid, indicating that NF-κB alone was insufficient and that the IFN-γ-induced p91 (STAT1) factor played an essential role. The biochemical basis of NF-κB induction by IFN-γ remains to be investigated.

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Regulation of INDO Gene by IFN-γ

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