Importance of the Two Interferon-stimulated Response Element (ISRE) Sequences in the Regulation of the Human Indoleamine 2,3-Dioxygenase Gene

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Indoleamine 2,3-dioxygenase (INDO) is the rate-limiting enzyme in the catabolism of the essential amino acid L-tryptophan. It is induced strongly in many cell lines following interferon-γ treatment. We report the cloning and characterization of the full-length human INDO promoter. This promoter is 1,245 base pairs long and includes two interferon-stimulated response elements (ISRE) separated by an approximately 1-kilobase sequence. The presence of these two ISREs is critical for maximum INDO promoter activity (50-fold induction). When the ISREs are present in two separate fragments cloned upstream of the chloramphenicol acetyltransferase (CAT) reporter vector, the INDO promoter activity drops significantly (7-fold induction). 5' end deletions of the wild type promoter sequence indicate that removal of the ISRE (ISRE1) at position −1126 reduces the induction level to approximately 25-fold. This activity does not change appreciably when the promoter is deleted down to position −241. Furthermore, site-directed mutagenesis of ISRE1 also decreases the promoter activity in a similar way. When ISRE1 is kept intact, deletion of the second ISRE (ISRE2) at position −111 leads to only 11-fold induction of the promoter. A similar result is obtained when substitution mutations are introduced in ISRE2. Deletion of a 748-base pair sequence between the two ISREs only shows a slight decrease in the INDO promoter activity. These data indicate that the two ISRE sequences are required for the full transcriptional induction of the interferon-γ-inducible human INDO gene. INDO activity is not induced in the hepatic cell line HepG2. An analysis of INDO-CAT activity in this cell line indicated that the lack of induction could be correlated with a truncated or unstable IRF-1. However, the levels of IRF-2, JAK2, and STAT 91 were similar in both ME180 and HepG2 cells.

Indoleamine 2,3-dioxygenase (INDO) is the rate-limiting enzyme in the catabolism of the essential amino acid L-tryptophan in mammalian cells. The enzyme is induced predominantly by IFN-γ (Taylor and Feng, 1991), although in human peripheral blood leukocytes both IFN-α and IFN-γ have been reported to induce the enzyme (Carlin et al., 1987). INDO plays an important role in the host response to intracellular protozoan infections (Pfefferkorn, 1984; Thomas et al., 1993), and tryptophan starvation as a result of INDO induction is involved in IFN-γ-induced cell death and apoptosis (Gupta et al., 1994). INDO activity is also down-regulated by the cytokine interleukin-4 (Musso et al., 1995).

The INDO gene was cloned by Konan and Taylor (1996) and has been shown to include two ISRE-like sequences, dominantly by IFN-γ. The 96-bp region also contains two copies of a 9-bp palindromic sequence similar to the GAS (IFN-γ activating site) sequence reported by others (Chon et al., 1992). The GAS sequence binds the IFN-γ-regulated DNA-binding factor STAT 91. Chon et al. (1995) have shown that this ISRE-like sequence is required for expression when the INDO promoter region is linked to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene. These researchers (Chon et al., 1995) concluded that both ISRE and one of the GAS-flanking sequences are necessary for this CAT expression. However, we have shown previously that INDO induction is not correlated with STAT 91 activation (Klein et al., 1994). Ozes and Taylor (1994) have shown previously by electrophoretic gel shift analysis that IRF-1 binds to this sequence and is an essential factor in the regulation of INDO expression. Kadoya et al. (1992) cloned another ISRE fragment. S1 mapping using a primer complementary to INDO cDNA (Kadoya et al., 1992) indicated a major transcription initiation site 33 nucleotides upstream of a putative translation initiation site, a position consistent with the sizes of the INDO message (approximately 1.8 kb) and of the INDO protein (42 kDa). A second ISRE sequence was found 99 bp upstream of that transcription site. These two reports identify different transcription start sites, the first 442 nucleotides 3' from ISRE1 (Dai and Gupta, 1990), the second approximately 100 bases from ISRE2. Thus the INDO promoter contains two ISRE regions of opposite polarity. In this paper we report that both ISRE regions are required for full induction of the INDO gene following treatment with IFN-γ and that deletion or mutation in either one of the two ISREs substantially decreases inducibility.

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1 The abbreviations used are: INDO, indoleamine 2,3-dioxygenase; IFN, interferon; bp, base pair(s); GAS, IFN-γ-activating site; CAT, chloramphenicol acetyltransferase; kb, kilobase(s); PCR, polymerase chain reaction; ISRE, IFN-stimulated response element; Wt, wild type; J-γI, β-galactosidase; GBP, guanylate-binding protein.
2 Konan, K. V., and Taylor, M. W. (1996) J. Interferon Cytokine Res., in press.
Cloning and Mutation Analysis of the Human INDO Promoter

MATERIALS AND METHODS

Cell Lines—ME180 (human cervical carcinoma) and HepG2 cells were routinely grown in Eagle’s minimal essential medium (Life Technologies, Inc.) supplemented with 10% bovine calf serum, streptomycin (100 μg/ml), and penicillin (100 units/ml). Human IFN-γ was a generous gift of Dr. S. L. Gupta (Hipple Cancer Research Center, Dayton, OH). pCAT enhancer vector (Promega, Madison, WI) allows the cloning of a eukaryotic promoter upstream of the CAT reporter construct.

DNA Isolation and Northern Blot Analysis—Total RNA was prepared by the CsCl step gradient method, according to Ausubel et al. (1988). For Northern blot analysis, 30 μg of total RNA was denatured, electrophoresed on a 1.2% agarose-formaldehyde gel, and transferred overnight to Zeta-Probe membrane in 20 × SSC. The membrane was prehybridized for 4 h at 41°C in a solution containing 25 mM KPO4 buffer, pH 7.4, 5 × SSC, 5 × Denhardt’s solution, 50 mg/ml salmon sperm DNA, and 50% formamide. Hybridization was performed at 41°C overnight with a prehybridization buffer containing the hybridization solution, 50% dextran sulfate, and approximately 2 × 106cpm/ml of a 32P-labeled probe. The membrane was washed three times at room temperature with 1 × SSC, 0.1% SDS, twice with 0.25 × SSC, 0.1% SDS, and then at 65°C for 30 min with 0.1 × SSC, 0.1% SDS. The blotted membrane was exposed overnight to an x-ray film for autoradiography.

PCR Cloning and Sequencing of the Wild Type INDO Promoter—Two oligonucleotide primers (upper primer, 5'-AGAATATTGCCATGGGATGATTGAGACCTTCATGAGCTTCTCT-3', containing an EcoRI site at the 5’ end, whereas the lower primer, 5'-AGATAGCATGTCGATGGTCGCCCTCCGATAAGATTTTGG-3', contains a BamHI site at the 5’ end) were designed such that they could amplify a 1.4-kb INDO fragment including the ISRE reported by Dai and Gupta (1990) and the major transcription initiation site (Kadoya et al., 1992). Genomic DNA was amplified for a total of 40 cycles. The PCR product was digested with both EcoRI and BamHI, purified on ELUTIP-D columns, and cloned into EcoRI/BamHI sites of pBS KS(−). Two independent PCR products (1.245 bp) were sequenced (Sanger et al., 1977) and were cloned upstream of the CAT reporter construct.

DNA Transfection—ME180 cells (5 × 104) were seeded in 100-mm tissue culture dishes and incubated at 37°C for 3 days to reach 50% confluence. The cells were washed with phosphate-buffered saline, fed with 4 ml of Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% NU-SERUM (Collaborative Biomedical Products, Bedford, MA) and incubated at 37°C for 2 h. 4 μg of CAT reporter plasmid DNA and 4 μg of pSV β-galactosidase vector (internal control) were co-transfected into ME180 cells by the DEAE-dextran method (Ausubel et al., 1988). The following day, some of the plates were treated with 2 μg/ml of IFN-γ and the NIH promoter activity measured.

CAT and β-Galactosidase Assays—Cell extracts for CAT and β-galactosidase assays were prepared using 1 × reporter lysis buffer (Promega). CAT activity was measured using a non-TLC method (Amer sham Corp.), while β-galactosidase activity was measured according to a protocol from Promega.

Deletion Analysis—A 1.245-bp HindII/Spel fragment containing the INDO promoter was inserted at HindII/XbaI sites in front of CAT structural gene in the pCAT enhancer vector. 10 μg of the HindII/XbaI pCAT vector was linearized at the HindII site, blunt ended with Klenow and diphosphoethane deoxyynucleotides (Promega) to protect the 5’-protruding end from cleavage by exonuclease III. The vector was then cleaved with EcoNI to create a 5’-protruding end for exonuclease III digestion. After phenol/chloroform extraction and ethanol precipitation, the DNA was dissolved in 25 μl of exonuclease III 1 × buffer (66 mM Tris-HCl, pH 8.0, 66 mM MgCl2). The DNA was warmed up to 22°C (the digestion temperature that allows 80-bp digestion/min), and 450 units of exonuclease III were added to the sample and mixed. 2.5 μl samples were removed at 1-min intervals into a previously prepared 7.5-μl S1 nuclease mix (172 μl of dideoxy H2O, 27 μl of S1 7.4 × buffer (0.3 M potassium acetate, pH 4.6, 2.5 M KCl, 10 mM ZnSO4, 50% glycerol), 60 units of S1 nuclease) on ice and mixed by briefly pipetting up and down. After all of the samples were taken, they were moved to room temperature for 10 min to allow S1 end to dialyze into the dehydrated strands. 1 μl of S1 stop buffer (50 μl of Tris, 0.05 μM EDTA) was added to each sample, and the mixture was heated at 70°C for 10 min to inactivate the S1 nuclease. 1 μl of Klenow mix (30 μl of Klenow buffer, 5 μl of Klenow enzyme) and 1 μl of the dNTP mix (0.125 mM each, dATP, dCTP, dGTP, and dTTP) were added to the remaining samples, and the mixture was incubated at 37°C for 8 min. The samples were then transferred to room temperature, 40 μl of ligase mix (790 μl of dideoxy H2O, 100 μl of dideoxy 10 × buffer (500 mM Tris-HCl, pH 7.6, 100 mM MgCl2, 10 mM ATP), 100 μl of 50% polyethylene glycol, 10 μl of 0.1% Triton X-100, 5 μl of T4 DNA ligase) for each sample, and the mixture was incubated at room temperature for 1 h. 10 μl of each sample was used for transformation of competent cells. Other deletions were obtained using restriction endonucleases.

Oligonucleotide-directed Mutagenesis of ISRE Sequences in the INDO Promoter—Site-directed mutagenesis of the ISRE at position −126 (ISRE1) was performed according to Kunkel et al. (1989). The INDO promoter was cloned into PBS KS(−) to obtain a single-stranded DNA template from a dur ‘ung’ Escherichia coli strain. A 30-nucleotide primer (5'-CTTGATAATTTGCTGACGTTTCTTACA-3') was designed in such a way that two substitutions in the ISRE residues of ISRE1 create a PstI site. The presence of the point mutations was checked by PstI restriction analysis and by DNA sequencing. A 260-bp INDO fragment that contains ISRE at position −111 was gel purified and used as a template for PCR mutagenesis of the ISRE2. Two oligonucleotide primers (upper primer, 5'-TTTACGACCGACTTCATTATC-3', and lower primer, 5'-AAATATCTCCCTTGGAAATGTTACCA-3') were designed such that the lower primer would introduce two point mutations (bold letters AC) in the ISRE2 sequence, creating a KpnI site. These mutations were confirmed by restriction digestion and sequencing.

Western Blot Analysis—ME180 cells and HepG2 cells were grown as described, harvested, washed, and resuspended in an appropriate volume of lysis buffer (0.5% Triton X-100, 10% glycerol, 50 mM Tris-HCl, pH 8, 200 mM NaCl, 0.1 mM sodium vanadate, 50 mM sodium fluoride, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mg/ml pepstatin, 0.1 mM benzamidine, and 0.5 mg/ml leupeptin). Samples were centrifuged at 2,000 rpm for 10 min at 4°C, and the supernatant was maintained at −80°C. 50 μg of protein was mixed with an equal volume of 2 × Laemmli buffer (4 ml of dideoxy H2O, 1 ml of 0.5 M Tris-HCl, pH 6.8, 0.8 ml of glycerol, 1.6% of 10% SDS, 0.4 ml of β-mercaptoethanol, and 0.2 ml of 0.5% bromphenol blue), electrophoresed on SDS-polyacrylamide gel, and transferred to nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) for 1 h at room temperature, washed for 10 min in TTBS (20 mM Tris, 500 mM NaCl, 0.05–0.3% Tween 20, pH 7.5), and incubated overnight with antibody to the respective protein. Following washing the membrane was incubated for 3 h with alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit IgG and the blot developed with enhanced chemiluminescence (Bio-Rad).

RESULTS

Cloning of the Wild Type INDO Promoter Sequence from ME180 Cells—The INDO promoter sequence including ISRE1 and ISRE2 is presented in Fig. 1A. A 1.245-bp fragment from the 5’ region of the INDO gene was synthesized by PCR and cloned upstream of the CAT reporter gene in the pCAT enhancer vector (Fig. 1B) and the resulting construct called wild type (WT). WT and pSV β-galactosidase (pSV-β-Gal) were co-transfected into ME180 cells by the DEAE-dextran method. The 1.4-kb fragment reported previously to contain the promoter signals (Dai and Gupta, 1990) was also transfected into ME180 cells for promoter activity comparison. When ME180 cells were transfected with WT construct and treated with IFN-γ for 24 h, the promoter as measured by the CAT reporter construct shows a 50-fold induction over the background (Fig. 1B). However, extracts from ME180 cells transfected with the 1.4-kb INDO fragment show only 4-fold induction (Fig. 1B) of the promoter. These results indicate that the 1.4-kb fragment does not contain all of the cis-acting elements required for full INDO promoter activity.

Role of the 855- and 390-Base Pair INDO Regulatory Sequences in the Induction of the INDO Promoter—The WT INDO promoter was cleaved with PstI to generate two fragments (855 and 390 bp) each containing an ISRE-like sequence. Both fragments were cloned upstream of a CAT reporter construct and transfected into ME180 cells. Following IFN-γ treatment, a low level of induction (7–8-fold), is observed with each fragment (Fig. 1B). These results indicate that neither the 855-bp nor the 390-bp fragment is sufficient for wild type INDO promoter.
The 1,245-bp INDO promoter was cleaved with PstI to remove an approximately 161-bp sequence, resulting in a 390-bp ISRE fragment. The remaining vector was religated to give the 855-bp ISRE fragment. The deleted 390-bp ISRE fragment was subcloned into the HindIII site of pCAT enhancer vector. The resulting construct was called Wt. The Wt was then digested with PstI to delete the 390-bp ISRE fragment. The remaining vector was religated to give the 855-bp CAT construct. The deleted 390-bp fragment was subcloned into the PstI site of pCAT enhancer vector to give the 390-bp CAT construct. CAT and β-Gal assays were performed as described under "Materials and Methods." Following transfection and IFN-γ treatment, the 1,245-bp INDO fragment shows 50-fold induction, whereas the other fragments show little activity (less than 10-fold induction).

These data also suggest that some cis-acting elements within the two fragments may be required for full INDO promoter induction or that both ISREs together are required for full activity.

### Role of the ISRE2 Sequence in the Induction of INDO Promoter—5' End Deletion Analysis of the INDO Promoter

Panel A, INDO promoter sequence including ISRE1 and ISRE2. P1 and P2 are the primers used to amplify and clone the wild type promoter. Also shown are the TATA box and the transcription (+1) and translation (ATG) start sites. The cloned sequence is approximately 1,245 bp. ISRE1 is equivalent to −449 to −435 of Chon et al. (1995). The promoter was first cloned into EcoRI/BamHI sites of pBS KS(−) for sequencing. Panel B, construction of the 390- and the 855-bp vectors. The 1,245-bp INDO promoter was cleaved with EcoRI/BamHI, blunt ended, and cloned in the blunt-ended HindIII site of pCAT enhancer vector. The resulting construct was called Wt. Wt was then digested with PstI to delete the 390-bp ISRE fragment. The remaining vector was religated to give the 855-bp CAT construct. The deleted 390-bp fragment was subcloned into the PstI site of pCAT enhancer vector to give the 390-bp CAT construct. CAT and β-Gal assays were performed as described under "Materials and Methods." Following transfection and IFN-γ treatment, the 1,245-bp INDO fragment shows 50-fold induction, whereas the other fragments show little activity (less than 10-fold induction).

Panel B shows the TATAbox and the transcription initiation (T) site of the INDO promoter. The distance between ISRE1 and ISRE2 in the INDO promoter is exactly 1,000 bp (95.2 turns of the helix). Typically, ISREs present in genes such as human tryptophan-tRNA synthetase (Frolova et al., 1993) and mouse guanylate-binding protein (GBP) (Briken et al., 1995) are separated by less than a 400-bp sequence. It was therefore of interest to examine if a reduction of the distance between the two ISREs would affect the INDO promoter induction. Deletion of a 748-bp EcoN1/PstI fragment (Δ748) between the two ISREs (Fig. 2) does not significantly affect INDO promoter induction (40-fold induction). These data indicate that the distance between the two ISREs is not critical and further demonstrate the importance of these cis-acting elements in the induction of the INDO gene.

Site-directed Mutagenesis of the ISRE1 and ISRE2 Sequences—Levy et al. (1988) have demonstrated that introduction of point mutations into the conserved T residues of the ISRE consensus sequence (NAGTTCTA/NTTTTNC) of IFN-α/β-inducible gene ISG54 abolishes or sharply reduces the promoter activity as measured by RNase protection assay. Two substitutions were therefore introduced in the T residues of the ISRE1 sequence (Fig. 3A) and the resulting construct called T-ISRE1. Transfection of T-ISRE1 into ME180 cells (Fig. 3B) also shows a decrease in the INDO promoter activity (24-fold induction). This result is similar to that obtained with a deletion of the ISRE1 sequence and strongly supports the hypothesis that ISRE1 is required for INDO promoter induction. Two substitution mutations were also introduced in the A residues of ISRE2 at position −111 (Fig. 3A) and the resulting construct called A-ISRE2. Transfection of the A-ISRE2 into ME180 cells (Fig. 3B) shows a sharp decrease (4-fold induction) in the promoter activity following IFN-γ treatment. This result is also similar to that obtained with the ISRE2 deletion mutants and confirms the hypothesis that ISRE2 is also critical for INDO promoter induction.
CAT activity following treatment with IFN-β is regulated by an ISRE-like element and GAS sequence found in the Wt INDO-CAT construct and treated with 500 units/ml IFN-β in HepG2 cells following treatment (Fig. 4A). Preliminary results from Northern analysis indicate that IRF-1 is unstable (or not synthesized for a long time) in HepG2 cells following treatment with IFN-γ (Fig. 4B).

**DISCUSSION**

Previous results have suggested that the INDO promoter is regulated by an ISRE-like element and GAS sequence found between positions –1126 and –1083 (Fig. 2) (Hassanain et al., 1993; Chon et al., 1995). Chon et al. (1995) have shown that when this element was linked to a thymidine kinase-CAT reporter gene there was a 9-fold induction of CAT expression following transfection of GM00637 cells. We find similar levels of CAT expression when ME180 cells are transfected with this construct. However, when the 1,245-base pair promoter region including two ISREs is placed upstream of the CAT construct, inducible levels are as high as 50-fold. This fragment contains approximately 855 bp of the 1.4-kb sequence reported by Dai and Gupta (1990) and around 390 bp of downstream sequence including the transcriptional initiation site of Kadoya et al. (1992). The 855- and 390-bp fragments each contain an ISRE sequence. When cloned upstream of the CAT reporter vector, these fragments are induced poorly (about 7-fold) by IFN-γ. 5' end deletions of the wild type promoter sequence indicate that removal of the first ISRE at position –1126 (ISRE1) leads to a 2-fold decrease in the promoter activity. Furthermore, site-directed mutagenesis of ISRE1 also drops the promoter activity in a similar way (24-fold induction). This activity does not change appreciably when the promoter is deleted down to position –241 (130 bp away from the second ISRE). When ISRE1 is kept intact, deletion of a 161-bp fragment including the second ISRE at position –111 (ISRE2) shows only 11-fold induction of the promoter.

Since the 1.4-kb fragment (Dai and Gupta, 1990) shares an 855-bp (5' end) DNA sequence with the wild type promoter, it was interesting to determine if the remaining 390 bp (3' end) of the promoter was sufficient to confer wild type activity. Transfection of the 390-bp fragment linked to the CAT reporter gene into ME180 cells alone shows only a 7-fold induction of the promoter. A similar induction (8-fold) is observed when the 855-bp fragment is transfected into ME180 cells. These results suggest that the cis-acting element(s) required for INDO promoter induction reside within or at the junction of the 855- and 390-bp fragments. 5' end deletions of the wild type promoter were thus performed using exonuclease III. Since in all of these deletion mutants the ISRE2 sequence is still present, it is likely that ISRE2 and/or flanking sequences are responsible for the 20–28-fold induction observed. To determine the importance of ISRE2, a 161-bp sequence including ISRE2 was deleted from the promoter. Transfection with the resulting ΔISRE2 vector shows that the promoter is induced poorly (11-fold).

The data from the deletion analyses suggest that ISRE1 and ISRE2 are two cis-acting elements critical for full INDO promoter induction. To confirm the importance of these elements, point mutations were introduced in either ISRE1 or ISRE2, and the effects of these mutations were analyzed. When T at positions –1124 and –1122 was changed to C and G, respectively, the resulting construct had a lower promoter induction (24-fold). This result is consistent with deleting ISRE1 and confirms the hypothesis that ISRE1 is indeed one of the INDO promoter cis-acting elements. In addition to deleting ISRE2, ΔISRE2 construct has also the X box (–114/-104) and Y box (–144/-135) removed. X and Y boxes are considered to be the essential components of the IFN-γ response region of major histocompatibility complex class II promoters (Benozzo and Mats, 1990; Dorn et al., 1987) and cannot be ruled out as part of INDO cis-acting elements. When point mutations were introduced in the ΔISRE2 sequence, the resulting construct showed only a 4-fold induction of the INDO promoter. These results indicate that the ISRE2 sequence, and probably not the X and Y boxes, is also required for induction of the INDO gene. Finally, deletion of a 748-bp EcoRI/PstI fragment between ISRE1 and ISRE2 shows that the distance between the two ISREs is not as important as the presence of these elements.
In an attempt to analyze the transcriptional induction of the human INDO gene, its promoter has been subjected to deletions and point mutation analyses. The results from these experiments strongly suggest that the INDO regulatory sequence spans at least a 1,245-bp fragment including two critical ISRE sequences. We hypothesize that following IFN-\(\gamma\) treatment, some newly synthesized factors bind to ISRE1 and ISRE2. INDO induction requires de novo protein synthesis since it is not induced in the presence of cycloheximide (Caplen and Gupta, 1988). This implies that STAT 91 is not directly involved in INDO induction because it is constitutively made and is activated after phosphorylation. However, the IFN-\(\gamma\)-inducible IRF1 protein is synthesized de novo. IRF1 has recently been associated with the induction by IFN-\(\gamma\) of the mouse GBP (Briken et al., 1995). Mouse GBP contains two ISRE sequences in its promoter region. Removal of the ISRE (Briken et al., 1995; Nicolet and Paulnock, 1994) close to the transcription start site abolishes GBP induction. Cotransfection of the GBP promoter construct and IRF1 cDNA up-regulates GBP promoter activity (Briken et al., 1995). The INDO gene has also two ISRE elements in its regulatory sequence and is likely to be transcriptionally induced by IRF1. Indeed, Ozes and Taylor (1994) have shown by Northern blot analysis that overexpression of IRF1 can induce an INDO message in ME180 cells. Furthermore, gel mobility shift assay indicates that IRF1 binds to ISRE1 of the INDO promoter. We show in this paper that in HepG2 cells, in which INDO activity is not induced, a truncated IRF-1 is made, and Northern analysis indicates that IRF-1 mRNA does not accumulate after 4 h of treatment with IFN-\(\gamma\), whereas in ME180 cells IRF-1 continues to accumulate up to 24–48 h after treatment. Together, these data argue for a role for IRF1 in INDO induction. We hypothesize that following IFN-\(\gamma\) treatment, some newly synthesized factors such as IRF1 (with possibly STAT 91 and/or other unknown factors) bind to ISRE1 and ISRE2. These transacting factors may come in contact through DNA looping at the complementary ISRE regions.

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