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# NOTES

## In Vivo Evidence of Interaction between Interferon-Stimulated Gene Factors and the Interferon-Stimulated Response Element

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**Constitutive and interferon-inducible DNase hypersensitive sites in vivo are located in interferon-stimulated gene promoters near sequences that specifically bind constitutive or interferon-inducible proteins in vitro. Induced sites and proteins are transient or maintained, depending on cell type. Interferon-stimulated gene transcription is transient or maintained in parallel.**

Interferon-stimulated-genes (ISGs) are transcriptionally activated when alpha interferon (IFN-α) binds to their cell surface receptors (1, 12, 23–25, 34). An IFN-stimulated response element (ISRE) was precisely defined by deletions and point mutations in the promoters of ISG15 and ISG54 (20, 25, 26, 34), and a similar sequence has been identified in other ISGs (3, 16, 17, 22, 32, 37, 38). Proteins that bind in vitro to the ISRE of ISG15 and ISG54 (20, 21, 26) and to other ISRE sequences (3, 16, 18, 32, 37) have been identified. Two of the DNA-binding activities are IFN-induced and are therefore candidates to be regulatory transcription factors (20, 21, 26); we term them IFN-stimulated gene factors (ISGFs). ISGF3 appears rapidly after IFN-α treatment, independent of protein synthesis in both HeLa cells and diploid fibroblasts. ISGF2 is also found in both cell types but is not induced until 90 min after IFN-α treatment; ISGF2 is not detected at all if protein synthesis is inhibited (26). Thus, ISGF3 correlates with the rise in transcription induced by IFN-α and so is proposed as an activating factor. Since ISG activation by IFN is typically transitory and the subsequent decline in transcription depends in part on new protein synthesis (23), the IFN-α-induced protein ISGF2 could be related to transcriptional suppression.

Models developed from gene transfer and in vitro binding results are supported in vivo by the localization of DNase hypersensitivity near the protein-binding sites in the endogenous genes. Such studies have been well reviewed (5, 6, 14, 30, 33, 41, 42). Constitutive DNase-hypersensitive sites are often found in genes that are poised for inducible transcription (2, 10, 13, 27, 39, 43, 45), and such constitutive sites can be enhanced or new sites can appear when such genes are actively transcribed. The *Drosophila* heat shock genes provide a well-documented case in which proteins bound at regulatory sites influence DNase hypersensitivity (36, 40, 44). Inducible sites have also been identified in the promoters of the interleukin-2 gene (39) and the IFN-γ-inducible gene *IP10* (27), among others. Examination of the relation among ISG chromatin structure, specific transcription factors, and transcription suggests that ISGFs act in vivo through ISRE.

**ISGs in WI38VA and Daudi cells are transcribed differently in response to IFN-α.** WI38VA (ATCC CCL 75.1) or Daudi cells sensitive to the effects of IFN-α (15) were grown in Dulbecco modified Eagle medium or RPMI 1640, respectively, each with 10% fetal bovine serum. Media were from GIBCO Laboratories, and serum was from Hyclone. Recombinant human leukocyte IFN-α A (Hoffmann-La Roche Inc.) was prepared at $10^6$ U/ml in Dulbecco modified Eagle medium.

The transcriptional response of ISGs to IFN-α treatment of WI38VA cells is completely analogous to that seen in nontransformed FS2 fibroblast cells (23, 24), HeLa cells (34, 35), or their own nontransformed WI38 precursors (data not shown). In all these cases, transcription peaks between 0.5 and 2 h after addition of IFN-α to cells. A decline to nearly basal levels after 6 to 12 h in the continuous presence of IFN-α partly depends on new protein synthesis. The level of the response is not maximal unless the cells are treated with at least 250 to 500 U of IFN-α per ml. As for all other responsive cells tested, induction of Daudi cell ISG transcription also occurs within 15 min after treatment with IFN-α. However, Daudi cells are distinguished by their response to as little as 5 U of IFN-α per ml, and run-on transcription assays (24) done with ISG and control probes previously described (31) showed that induction of the ISGs did not decline at later times but remained high after 6, 12, or 20 h in the continuous presence of IFN-α (data not shown). This result was consistent with the persistence of induced ISG mRNA in Daudi cells (21, 29).

**The presence of promoter-binding factors parallels transcription in each cell type.** We wished to compare the presence of ISGF in WI38VA and Daudi cells particularly, because of the prolonged IFN-α-induced ISG transcription in Daudi cells. Each cell type was treated for various lengths of time with IFN-α, and nuclear extracts were prepared essentially as described elsewhere (4). Some Daudi cell samples were also treated with cycloheximide before extracts were prepared. Specific DNA-binding proteins were then detected as previously described (26) with the mobility shift assay (11). Extracts from WI38VA cell nuclei were essentially identical to those from HeLa cells (26) or other fibroblasts. ISGF1 was present before and after IFN-α treatment. Both ISGF2 and ISGF3 were induced by IFN-α. ISGF3 had disappeared after 6 h of treatment. ISGF2 was much reduced after 6 h and was gone after 24 h of treatment (data not shown).

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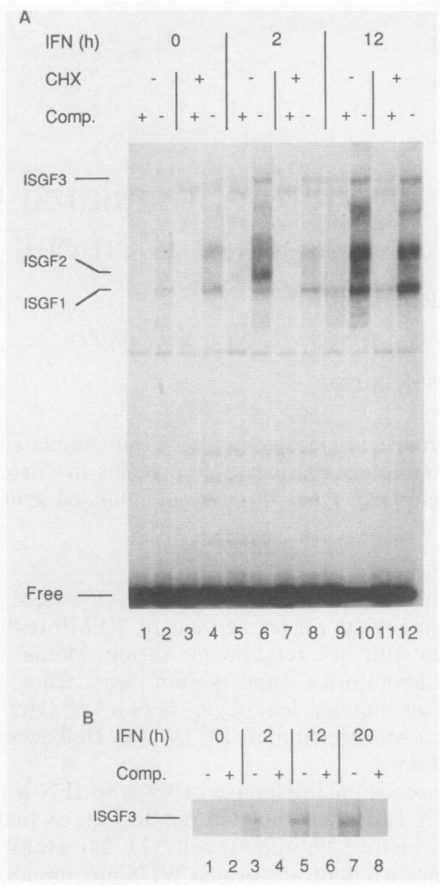


FIG. 1. Constitutive and IFN-α-induced nuclear factors, ISGFs, from Daudi cells binding to the ISRE regulatory element of IFN-α-stimulated genes. (A) Cultures of untreated Daudi cells or cultures treated with 100 U of IFN-α per ml for 2 or 12 h were divided. One sample of each received 25 μg of cycloheximide (CHX) per ml 2 h prior to addition of IFN-α or 2 h prior to harvest for the untreated culture. Nuclear extracts were made from all samples with 0.4 M NaCl and assayed with an ISG15 promoter fragment previously shown to bind to ISGFs via the ISRE sequence (20). Lanes 1, 3, 5, 7, 9, and 11, Reactions with specific ISG15 competitor DNA (Comp.); lanes 2, 4, 6, 8, 10, and 12, reactions with nonspecific competitor DNA only. The mobility of the free probe and the shifted complexes of the probe with ISGF1, ISGF2, and ISGF3 are indicated. Observations made during purification of ISGF1 and ISGF2 indicate that the complexes migrating between those indicated for ISGF2 and ISGF3 are higher-order structures that result from binding of ISGF1 or ISGF2 to the ISG15 promoter fragment (Pine, unpublished data). (B) Daudi cell nuclear extracts were prepared with 0.2 M NaCl after treatment with 25 U of IFN-α per ml for the indicated time. Either nonspecific (lanes 1, 3, 5, and 7) or specific (lanes 2, 4, 6, and 8) competitor DNA (Comp.) was included in the assay. Only the complex formed between the probe and ISGF3 is shown.

Assay of the Daudi cell extracts (Fig. 1) revealed one very important result. Like ISG transcription, induction of ISGF3 did not decline with time. As in other cells, ISGF2 was induced and then declined in Daudi cells, and at any time its presence required protein synthesis. Curiously, ISGF1 seems to increase after long treatment of Daudi cells. The additional bands present after long treatment result from this high concentration of ISGF1 and perhaps ISGF2 as well (see legend to Fig. 1).

**IFN induces DNase hypersensitivity at the ISRE in vivo.** To detect features of chromatin structure that could be attributed to ISGF binding in the nucleus, DNase I hypersensitivity in the vicinity of the ISRE was examined. At different times after addition of IFN-α to cells, nuclei were recovered by Dounce homogenization and centrifugation, washed, and treated with buffer alone or with increasing amounts of DNase I for 20 min at 30°C. DNA was extracted, and hypersensitive sites were detected essentially as described elsewhere (43) after restriction with *Sst*I or *Taq*I and electrophoresis on 1.2% agarose gels.

DNase-hypersensitive sites of ISG15 in WI38VA cells (Fig. 2A) were centered near positions +10 and −110, relative to the start of transcription, before IFN-α treatment (Fig. 2A, lanes 1 through 4). After 2 h of treatment with IFN-α, hypersensitivity at −110 was diminished, while the region around −170 had become hypersensitive (Fig. 2A, lanes 5 through 8). Finally, 24 h after addition of IFN-α, hypersensitivity had returned to the constitutive pattern (Fig. 2A, lanes 9 through 12). Figure 2B shows that ISG15 in Daudi cells also had constitutive DNase I-hypersensitive sites (near +1 and −120) and that the zone around −120 extended to −160 after 2 h of IFN-α treatment (Fig. 2B, lanes 4 through 6). However, unlike that of WI38VA cells, this pattern was maintained or even accentuated in Daudi cells after prolonged treatment with IFN-α (Fig. 2B, lanes 7 through 9). Figure 2C presents the data schematically for both cell lines.

In the ISG54 promoter region (Fig. 3), weak, constitutive DNase I-hypersensitive sites were seen in WI38VA cells (Fig. 3A) near −40, −160, and −530, relative to the start of transcription (Fig. 3A, lanes 1 through 4). The sites flanking the ISRE, near −50 and −170, were distinctly induced by 2 h of IFN-α treatment (Fig. 3A, lanes 5 through 8). As for ISG15, the hypersensitivity returned to basal levels in WI38VA cells 24 h after addition of IFN-α (Fig. 3A, lanes 9 through 12). In Daudi cells (Fig. 3B), ISG54 had constitutive DNase-hypersensitive sites around −60, −180, and −620 (Fig. 3B, lanes 1 through 3). The sites around −180 and −620 had enhanced hypersensitivity relative to the site at −60 after 2 h of IFN-α treatment (Fig. 3B, lanes 4 through 6); again, the induced hypersensitivity persisted in the Daudi cells even 20 h after addition of IFN-α (Fig. 3B, lanes 7 through 9). The extended hypersensitive zone downstream from −60 was lost after prolonged transcriptional activation of ISG54 in Daudi cells. Figure 3C shows the results schematically.

Because IFN-α-stimulated transcription is prolonged in Daudi cells but goes through the typical cycle in WI38VA cells, we were able to correlate chromatin structure with the presence or absence of ISGF3 and ISGF2 and compare this with the ISG transcriptional profiles. The consistent presence of ISGF3 when ISGs are transcribed, the cycloheximide resistance of both IFN-α-induced ISGF3 formation and ISG transcription, and the invariant absence of ISGF3 when ISGs are not expressed (as described above) (21) definitely mark this factor as the best candidate for an IFN-α-dependent ISG activator. The hypothesis that ISGF2 is a repressor can be maintained only with the provision that ISGF3 can override such activity. In several independent experiments, prolonged IFN-α treatment always increased levels of ISGF1 and the larger complexes formed from ISGF1 or ISGF2 in Daudi cells but not in any other cell type. The role of ISGF1 in ISG transcription is still uncertain.

The hypersensitive-site assays were performed to gain evidence that the ISRE of endogenous ISGs might have different bound proteins as part of its chromatin structure before and after IFN-α treatment. One noteworthy result is

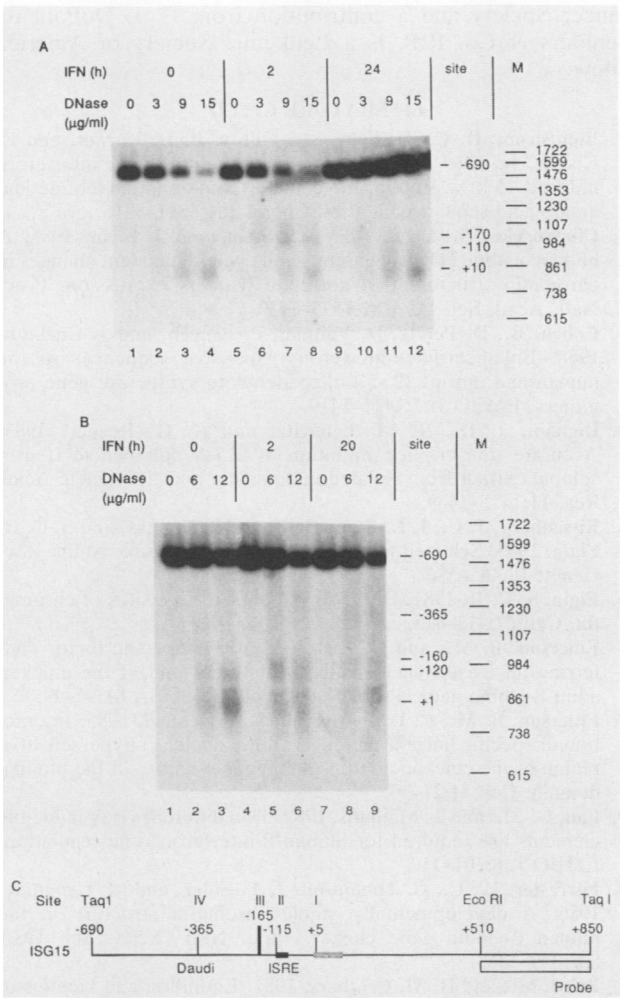


FIG. 2. Comparison of ISG15 constitutive and IFN-α-induced DNase-hypersensitive sites in WI38VA and Daudi cells at different times after IFN-α treatment. (A) WI38VA cells were treated with 500 U of IFN-α per ml for the time shown. Nuclei were isolated and treated with the indicated concentrations of DNase, and then DNA was extracted, digested with *Taq*I, electrophoresed, blotted, and probed with a radiolabeled ISG15 *Eco*RI-*Taq*I fragment (+510 to +850). Fragment sizes (base pairs) were calculated with the equation obtained from linear regression analysis of the marker mobilities ($R^2 > 0.99$), and the centers of sites were then estimated on the basis of known location of the probe within the gene. Resolution in the size range of the promoter was very good, and the centers of the DNase-hypersensitive sites were reproducibly mapped to within ±10 base pairs. The uppermost site is the full-length restriction fragment detected by the probe. The markers (M) used were 1-kilobase and 123-base-pair ladders (Bethesda Research Laboratories) labelled with $^{32}P$ and mixed with 20 μg of sheared salmon sperm DNA prior to electrophoresis. (B) Daudi cells were treated with 250 U of IFN-α per ml for the time shown. The analysis was performed as described for panel A. (C) The results illustrated by panels A and B are depicted schematically. Restriction sites and DNase-hypersensitive sites I through IV are shown above the horizontal line. The induced hypersensitive site is marked by the heavy vertical line. The positions given for the hypersensitive sites represent the centers of the analogous pair of sites in the two cell lines. A site observed in only one cell line is shown with that cell line designated underneath. The ISRE (■) and the first exon (▩) are shown. The subcloned fragment used as a probe (restriction sites and ▭) is indicated.

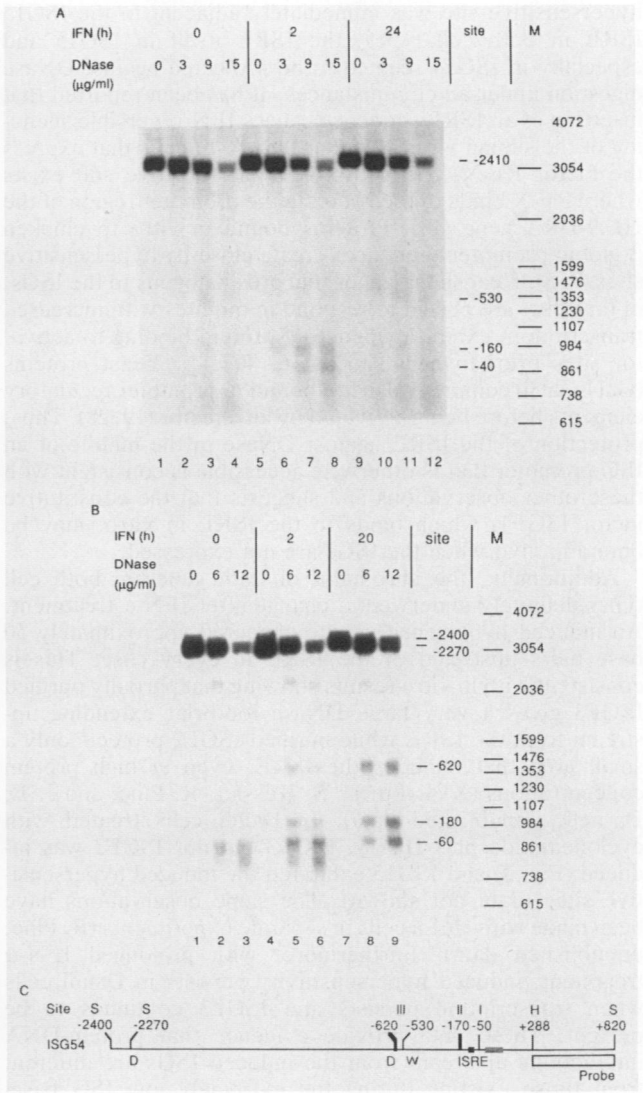


FIG. 3. Comparison of ISG54 constitutive and IFN-α-induced DNase-hypersensitive sites in WI38VA and Daudi cells at different times during IFN treatment. (A) WI38VA cell DNA from the same experiment as that described in the legend to Fig. 2A was digested with restriction endonuclease *Sst*I, electrophoresed, blotted, and probed with a radiolabeled ISG54 *Hin*dIII-*Sst*I fragment (+288 to +820). Hybridization to fragments of 2- to 3-kilobase pairs generated by DNase digestion is due to a unique homology between sequences from +410 to +580 in the probe and the region from −720 to −225 upstream of the first exon (Pine, unpublished data). Markers (M) and fragment size estimation were as described in the legend to Fig. 2A. (B) Daudi cell DNA from the same experiment as that described in the legend to Fig. 2B was analyzed as described for panel A. The large fragments which extend from the upstream end of the intact *Sst*I fragment to the sites of DNase cleavage are again visible. In addition, an *Sst*I restriction-fragment-length polymorphism of ISG54 is shown by the doublet at −2270 and −2400. The same polymorphism is observed with DNA from FS2 cells, while HeLa cell DNA has only the site at −2400 (Pine, unpublished data). (C) A schematic diagram of the results is organized as described in the legend to Fig. 2C. Restriction sites: S, *Sst*I; H, *Hin*dIII. Sites unique to Daudi cells (D) or to WI38VA cells (W) are indicated.

that before IFN-α treatment (when only ISGF1 was present), both ISG15 and ISG54 promoter regions had hypersensitive sites near the ISRE in both WI38VA and Daudi cells. Another key observation is that though a constitutive

hypersensitive site was immediately adjacent to the ISG15 ISRE in both cell types, the ISRE itself in ISG15 and especially in ISG54 seemed to be protected against DNase digestion under all circumstances. It has been reported that insertion of an ISRE sequence causes IFN-reversible silencing of the simian virus 40 enhancer (9). In cells that express the factor RF-X, a constitutive hypersensitive site exists where RF-X binds to the X box in the promoter region of the HLA-DRA gene (13). Proteins bound in vitro to chicken β-globin promoter sequences create close-by hypersensitive sites (7, 8). Heat shock genes that are analogous to the ISGs, in that they are poised to respond in minutes with increased transcription, exhibit evidence of protein binding to activator sites prior to heat shock (36, 40, 44). Yeast proteins (Gal4-Gal80 complex) also are bound to promoter regulatory elements before being activated by an effector (19, 28). Thus, protection of the ISRE against DNase in the middle of an ISG promoter that is otherwise accessible is consistent with these other observations and suggests that the constitutive factor ISGF1, which binds to the ISRE in vitro, may be bound in vivo when the ISGs are not expressed.

Additionally, the chromatin of both genes in both cell types definitely underwent a change after IFN-α treatment. An induced hypersensitive site appeared approximately 50 base pairs upstream of the ISRE in every case. This is consistent with in vitro results showing that partially purified ISGF3 gives a very large DNase footprint extending upstream from the ISRE while purified ISGF2 protects only a small area that includes the ISRE, even at high protein concentrations (X. J. Fu, D. S. Kessler, R. Pine, and J. E. Darnell, unpublished data). In Daudi cells treated with cycloheximide plus IFN-α, ISGF3 but not ISGF2 was induced (Fig. 3) and ISG15 exhibited the induced hypersensitive sites (data not shown). The same observations have been made with HeLa cells in separate experiments (R. Pine, unpublished data). Furthermore, with prolonged IFN-α treatment, induced hypersensitivity persists in Daudi cells when transcription persists and ISGF3 continues to be present. These data provide evidence that protein-DNA interactions upstream from the induced ISGs are different from those existing during the extremely low ISG basal transcription and suggest that the induced DNase I-hypersensitive sites are attributable to ISGF3, despite the possible presence of some ISGF2 late after IFN-α treatment.

After prolonged IFN treatment of Daudi cells, changes in hypersensitivity near the cap site were seen clearly for ISG54 and may have occurred in ISG15. There may also have been diminished cleavage by DNase near the cap site of both genes in WI38VA cells when transcription neared its peak, though the change was quite modest. These results suggest protection from DNase due to continual reinitiation of transcription. At present we have no information on the DNA regions between −300 and −600 that are hypersensitive. We do know that 122 nucleotides of upstream sequence will confer IFN-α-induced transcription (25, 26, 34), but we have not formally proven that the programmed transcriptional decrease occurs with the 120-nucleotide segment. Further studies will seek to understand the significance of the far-upstream hypersensitive sites. With purified proteins and the genes that encode them, we should gain a more detailed understanding of the interactions of the ISGF and ISRE in regulation of ISGs.

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