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INTERFERON-INDUCED EARLY CHANGES IN NUCLEAR PROTEIN INTERACTIONS WITH THE INTERFERON CONSENSUS SEQUENCE

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A number of genes are transcriptionally regulated by interferons via a cis acting regulatory element (interferon consensus sequence). After incubation with HeLa cell nuclear extracts, a synthetic 29 mers probe representing the consensus was retarded as a single band whose amount increased after the addition of IFN to the cells. DNase I footprinting showed that a region of the non coding strand was protected differentially upon interferon treatment. After nuclear protein blotting and probing with the oligonucleotide, two proteins (114 and 50 kDa) of the desired specificity were revealed. Their absolute and relative amounts do not appear to be dependent of the IFN treatment.

The effects of IFNs are exerted through their interaction with cell surface receptors specific for either type I ($\alpha\beta$) or II (γ) IFNs (1-3) but the biochemical consequences of this interaction at the plasma membrane are still poorly understood (4-6). One of the earliest event in IFN action is the transcriptional activation of a number of genes (7-13). For some of these IFN-inducible genes, the presence of a conserved region of about 30 bp generally designed as IFN consensus sequence (ICS) has been observed (14). Since this initial observation, a number of other genes whose expression is modulated by IFNs have been shown to possess this sequence or subsets of this motif (15-18). Therefore, a shorter new consensus element (13-18 bp) had been defined (ISRE, for Interferon Stimulated Responsive Element)(19-22).

We aimed at pinpointing protein(s) interacting with the ICS and their eventual modifications upon IFN treatment irrespective of the gene context, since they are the most likely common target for signalling pathway(s) activated by the initial interaction of IFNs with their cognate receptors. Experiments presented here have been performed on a simple model consisting of a 29-mer double-stranded oligonucleotide representing a slightly modified version of the originally described ICS (14). Our study included all three available approaches for studying DNA-protein interactions, i.e. gel retardation assay, DNase I footprinting, and identification of gel fractionated nuclear proteins with labeled oligonucleotides

Abbreviations : IFN, interferon; ICS, Interferon Consensus Sequence; ISRE, Interferon Stimulated Response Element; DNase I, deoxyribonuclease I.

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MATERIALS AND METHODS

Cell culture : Nuclear extracts from Hela cells were prepared according to Dignam et al.(23). Type I IFN (1.2 x 106 IU/mg) was donated by Meyrieux (Lyon, France). and recombinant type II IFN (107 IU/mg) was given by Roussel-Uclaf. For cell treatment, IFNs were used at 500 U/ml. Proteins were assayed according to Bradford (24).

Oligonucleotides : Two complementary 29 mer oligonucleotides were produced on a Biosearch DNA synthesizer . The sequence chosen is the following :

5'TTCACCACCTCCGCAGTTTCTCCTCTACT 3'
3'AAGTGGTGGAGGCGTCAAAGAGGAGATGA 5'

Oligonucleotides were purified by electrophoresis and labeled using (α-^{32}P) ddATP (3000 Ci/mM) with terminal transferase (Pharmacia).

Gel retardation assay : Nuclear extracts were incubated with 10,000 cpm of the probe (0.03 - 0.1 ng) at 30°C under 20 μl in a medium containing : 20 mM Hepes-KOH pH 7.9, 10% (v/v) glycerol, 0.25 mM dithiothreitol, 0.1 mM EDTA, 50 mM KCl, 0.5 mM of each ATP, CTP, GTP, UTP, 2-10 mM $MgCl_2$, 0.5 μg E. Coli DNA, 0.25 - 0.5 μg poly (dI).poly(dC), and 3-6 μg of nuclear extract. Bound and free probe were separated on polyacrylamide gels (acrylamide/bisacrylamide : 60/1) containing 6% (v/v) glycerol,10 mM Tris HCl pH7.4 and 2 mM $MgCl_2$ using 10mM Tris HCl pH7.4 and 2mM $MgCl_2$ as a running buffer.

DNase I footprinting experiments : Incubations were performed as described above except that the amount of nuclear extract was increased to 12 μg in order to allow the binding of most of the probe. Samples were then incubated for 5 min at 30°C with 200 μg/ml DNase I (Sigma) and analysed on a sequencing gel (15% acrylamide) using a thickness gradient (0.35 to 0.70 mm).

Protein blotting and probing with the ICS : Nuclear extracts (60 - 100 μg/well) were precipitated with acetone to remove KCl and proceeded for SDS-PAGE analysis (12.5 % acrylamide) as described by Laemmli (25).After electrotransfer to nitrocellulose, membranes were incubated for 2 h in 50 mM NaCl, 10 mM Tris HCl pH7.4, 10 mM EDTA, 0.1 mM phenyl methyl sulfonide chloride and 5 % (w/v) non fat dry milk. Filters were then rinsed for 5 min in the buffer D described by Dignam diluted twice and hybridized with the probe (105 cpm/ml) for 3-4 h. Filters were then rinsed twice for 2 min , dried and autoradiographed.

RESULTS

Specificity of the interaction of the ICS with HeLa Cell nuclear extracts

The sequence synthetized was derived from the ICS found in the following genes : HLA-DR, HLA class I (2 genes), metallothionein (14), C202 (18), 56 kDa (all 3 ICS) (11) and H-2L^d (26). The oligonucleotide was retarded as a single band during electrophoresis after incubation with nuclear extracts (Fig.1). An excess of unlabeled ICS added together with the probe abolished the signal. The addition of an excess unlabeled oligonucleotide after preincubation of the probe with the nuclear extract did not lower significantly the intensity of the retarded band, even after 60 min. Thus the detected interaction was stable(not shown).

In order to evaluate the specificity of the interaction and/or the number of proteins able to interact with the ICS, the amount of nuclear extract was varied from 1.5 to 15 μg. The amounts of competitor E. Coli DNA and poly(dI) poly(dC) at the highest concentration tested exceeded by 15,000 fold that of the probe (Fig. 2). For all these conditions, a single band was retarded with a similar mobility shift and the unlabeled ICS concentration range eliciting the displacement of the probe was similar.

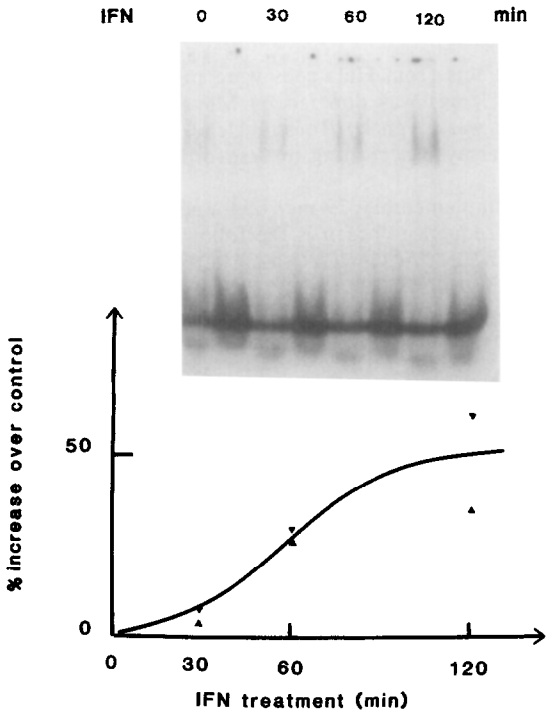

Fig. 1 : Effects of IFN treatment on the capacity of nuclear extracts to bind ICS.
Cells were grown as usual and treated with 500 U/ml of both $\alpha\beta$ and γ IFNs for the indicated periods of time. Protein concentrations of nuclear extracts were adjusted to identical values. Each extract was assayed for ICS binding in the presence (even lanes) or absence (odd lanes) of 500 nM unlabeled ICS. After autoradiography (upper panel), the retarded bands were excised and counted. The values obtained from two independent experiments are plotted in the lower part of the figure.

None of the strands constituting the ICS was able to compete for ICS binding, nor a series of 9 ICS-unrelated double-stranded oligonucleotides with lengths ranging from 16 to 25 mer when tested at concentrations up to 1 μM (not shown). An estimation of 10,000 binding sites per cell can be made. If the ICS binding protein has a molecular weight of 100 kDa (vide infra), it would represent 0.01 % of the proteins in the extract which is a reasonable value (27).

Effect of IFN cell treatment on the ICS/protein interaction

Nuclear extracts prepared from cells which have been treated with IFN for 0.5, 1 and 2h, or left untreated, exhibited a similar gel retardation pattern and unlabeled ICS was able to fully compete for binding (Fig.1). A 50 % increase in the amount of the retarded probe was observable 2 h after the onset of IFN treatment. Such results were confirmed using a dot assay (not shown) in which the reaction mixture was spotted on nitrocellulose membranes retaining DNA-protein complexes but not unbound DNA probes (28). A 75 % to 100 % increase in binding capacity could be detected, with a half maximum effect being obtained within 0.5 h.

IFN-induced differential ICS/proteins interaction identified by DNase I footprinting

Nuclear extracts from untreated cells protected strongly 18 out of the 30 bases of the bottom strand of the ICS from DNase I digestion (Fig.3). A first protected region included

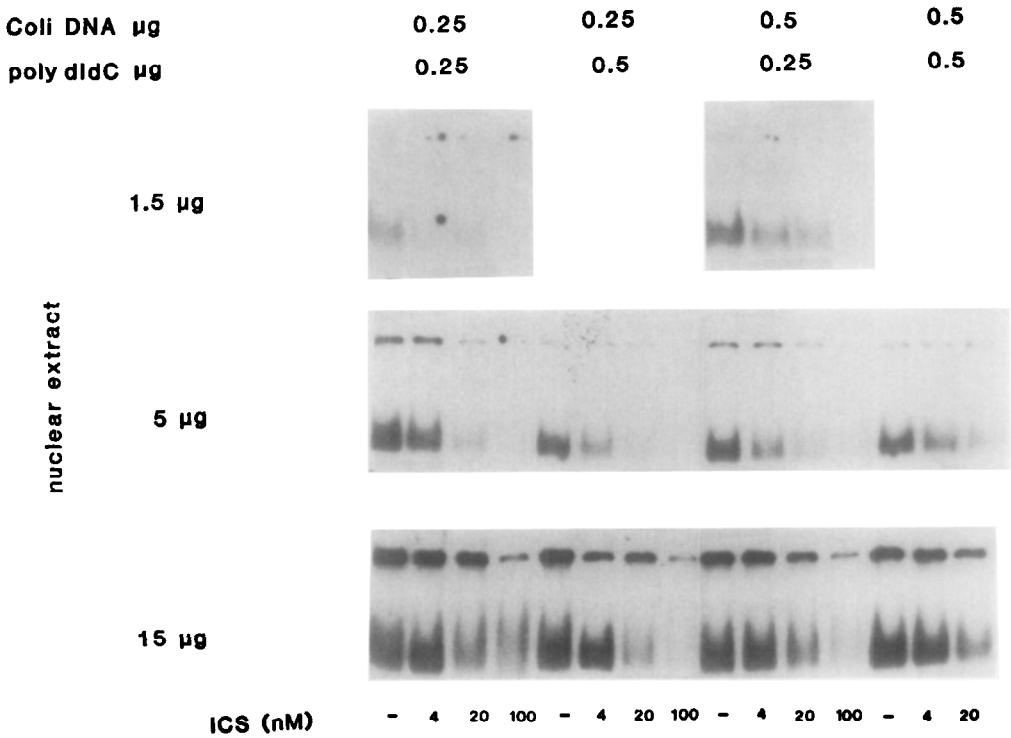

Fig. 2 : Lack of competition by non specific DNA competitor for nuclear protein(s) binding to the ICS probe : effects of nuclear protein concentration.

To the indicated amounts of nuclear extracts were added fixed amounts of E. Coli DNA and poly(dI)-poly(dC) together with unlabeled ICS as described in the figure. For convenience, only the retarded band is shown. In none of the conditions used, was any other retarded band apparent except at high protein concentrations, for the material stacked in the wells. When using 15 µg nuclear extract most of the probe was retarded.

the 5' end of this strand (Box1) suggesting that for at least some of the natural genes, the extent of the protection could be further extended. With nuclear extracts from cells which had been treated with type I or type II IFNs, the extent of the protection on the 5' end of the non coding strand was diminished. A second box (GGAG) protected from DNase I digestion was apparent. No differential pattern due to IFN treatment could be detected on the opposite strand albeit a protection of the 3' end did exist.

The DNase I digestion patterns observed with an excess unlabeled ICS indicated that the mere presence of the nuclear extracts was not responsible for an altered (differential) specificity of DNase I action (Fig.3, right part, first four lanes). In addition, none of these two protected regions resulted from a reduced sensitivity of the probe to DNase I (Fig.3, right part, last four lanes).

Evidence for two nuclear proteins interacting with the ICS

After gel electrophoresis and transfer to nitrocellulose, the ICS can bind to two distinct proteins whose molecular weights are 114 kDa + 7 and 50 kDa + 6 (n = 9) (Fig. 4). Increasing the probe concentration did not allow the detection of other ICS binding proteins (lanes 1 and 3) while unlabeled ICS prevented their detection (lanes 2 and 4). On the

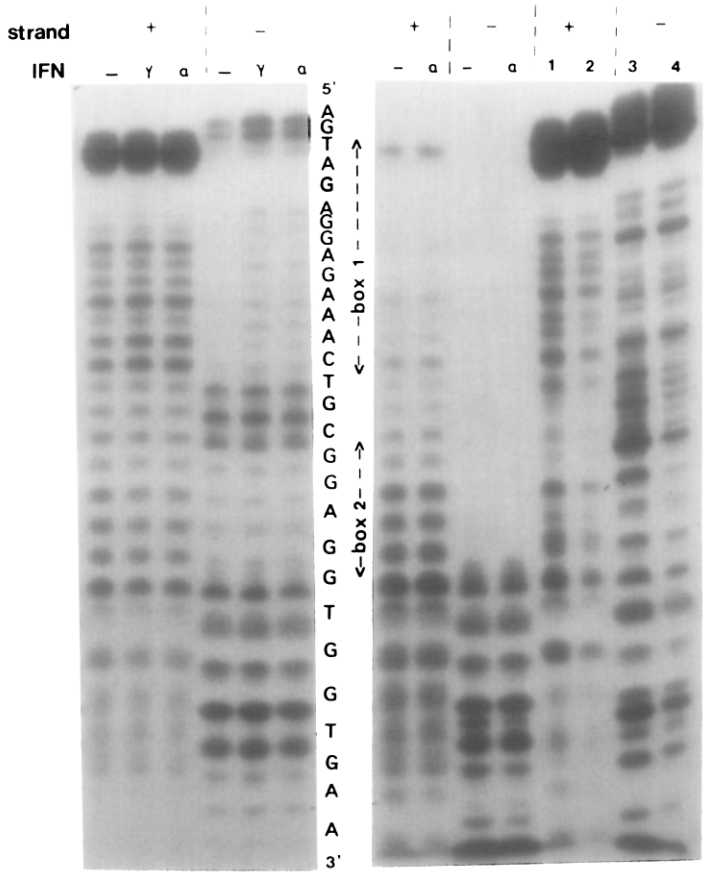

Fig. 3 : DNase I footprinting of protein binding to the ICS : effects of IFN cell treatment.
Left part : Footprinting reactions were performed using identical amounts of nuclear extracts from either untreated cells, or from cells treated with either $\alpha\beta$ IFN or γ IFN. The protected regions are indicated as box 1 and 2. Only the sequence of the bottom strand is indicated.
Right part :
-first four lanes : experimental conditions were those described for the left part of the figure except that 500 nM unlabeled ICS was present during the incubation .
-last four lanes : Nuclear extracts were omitted from the incubation mixture. DNase I concentrations used were one third (lanes 1 and 3) and one tenth (lanes 2 and 4) those used for the footprinting reaction. The incubation with DNase I was for 2 min.

contrary, the addition of 1 μM of an oligonucleotide unrelated to the ICS did not alter the binding (lane 5). No difference in the number of detected bands, as well as in their relative amount could be detected as a function of time upon type I and type II IFN addition, either alone or in combination.

DISCUSSION

A synthetic oligonucleotide representing a compromise for some of the reported ICS allows the direct demonstration of an interaction of protein(s) with a minimal sequence, eliminating for the sake of simplification the potential effects of cis acting elements. Such a system might allow to define the effectors which could directly affect the interaction under

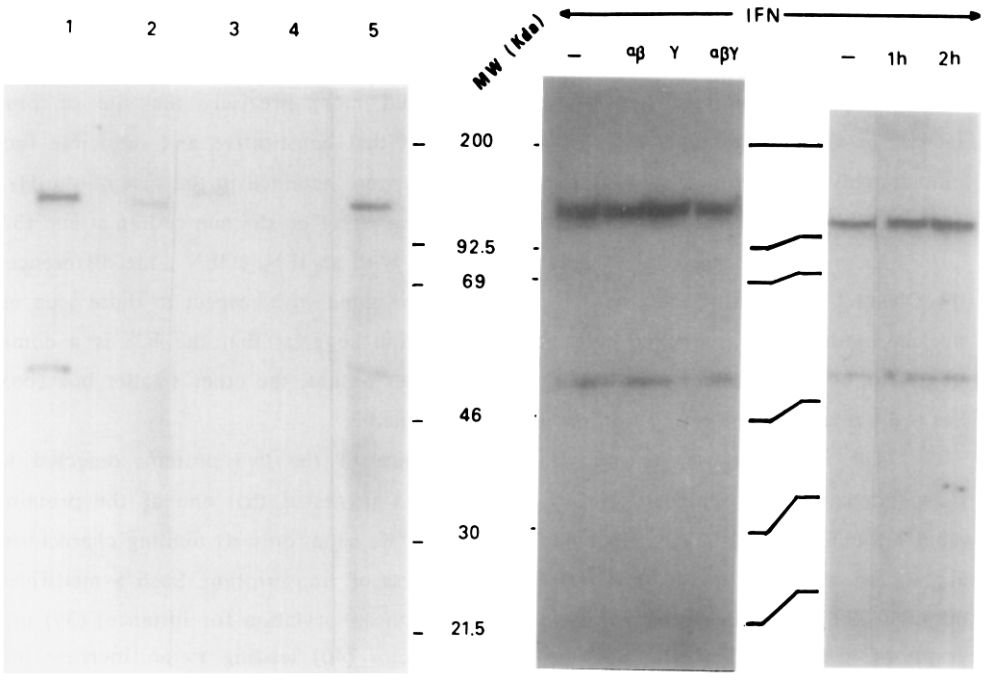

Fig. 4 : Nuclear extracts protein blotting : probing with the ICS.
Left part: Specificity of the interaction
Protein blots were hybridized with either 2 x 10^5 cpm (lanes 1,2 and 5) or 4 x 10^4 cpm (lanes 3 and 4) labeled ICS/ml. An excess (500 nM) of unlabeled ICS (lanes 2 and 4) or of a 22 mers oligonucleotide unrelated to the ICS (lane 5) was eventually added during the hybridization.
Center part : Effects of the IFN treatment on the protein pattern
Identical amounts of nuclear extracts prepared from untreated cells (-), or cells treated for 1h with $\alpha\beta$ IFN, γ IFN or their combination were loaded on the gel.
Right part : Time course of IFN action.
Nuclear extracts were prepared from untreated cells (-) or from cells treated with 500 U/ml of both $\alpha\beta$ and γ IFNs.

study. However, the observed effects may be altered either qualitatively and quantitatively due to the degeneration of the consensus.

The ability of proteins to recognize fragment of genes containing the ICS and/or the ISRE has been demonstrated (19, 20, 29-34). Some of these factors preexist to IFN action (30,35); others are induced upon IFN addition and are potentially part of a feed-back regulatory mechanism. A simplification of the system has been attempted using oligonucleotides representing the 5' upstream region of H-2L^d (32), 6-16 (36), 9-27 (36,37), ISG 54 and 15 (38) genes. A rigorous comparison of our results with those described in the litterature is difficult to make since the biological material, the experimental conditions and the probes used are different. We confirm the increase ,albeit low and labile, in the ability of the HeLa cell nuclear extract to recognize such a sequence upon IFN cell treatment (29,30,33,34,38). This observation suggests that either the protein(s) interacting with the ICS is present in a higher amount or that the strength of the interaction is enhanced. However under the incubation conditions we used, we never detected a second or a third band

suggestive of an interaction with the ICS of other protein in the gel retardation assay. Such a pattern (as observed by several authors (29,32,34,38) implies that the binding of the nuclear factors detected were mutually exclusive.

Methylation interference assays have located more precisely the site of protein interaction(s) with the ISRE (20,32,34) and showed that constitutive and inducible factors bind roughly to the same region. Similar conclusions were obtained in the case of the H-2L^d gene, the extent of the protein-DNA interaction being larger on the non coding strand (32).

In this paper, whether the cells were treated with ab IFN, c IFN , the differences in the DNase I footprinting patterns observed were the same with respect to those seen using nuclear extract from untreated cells. This observation suggests that the ICS is a common target for both type I and type II IFNs (20). Whether or not, the other smaller box (box 2) has to be related to the IFN action has to be determined.

The molecular weight and relative abundance of the two proteins detected were independent on cell treatment with IFN. It may be suggested that one of the proteins is modified upon IFN action ,not its amount, in such a way that only its binding characteristics are altered with yet unknown effects on the process of transcription. Such a modification might be the consequence of a biochemical event (phosphorylation for instance) (39) or the result of an interaction with another (protein) factor (40) leading to an increase in the binding capacity observed in the gel retardation experiments.

The number of factors involved in the interaction described could lead to a fine regulation of the IFN response. Depending on the individual sequence for each gene controlled by IFN, these interactions could take place to different extent. In addition the potential existence of a feed back regulator interacting with part of the ICS and cis acting element has to be defined (32,34,38). Such a system could explain the variety of the IFN responses observed either as a function of the type of IFN used, or as a function of time. It may also account for some of the potentiation effects observed when using both type I and II IFNs on some responses (7,41,42). Such a model appears suitable to investigate the nature of the second messengers of IFN action.

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