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Enhancer functions and *in vitro* protein binding of native and mutated interferon-responsive sequences

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ABSTRACT

Mutants of the Interferon responsive sequence (IRS) of the mouse and human (2'-5') A synthetase (moE-IRS and hE-IRS) were tested for their Interferon (IFN)-inducible enhancer functions and for protein binding *in vitro*. Two complexes R1 and R3, were formed specifically with the hE-IRS. R3 migrated much faster and was about ten times more abundant than R1. R1 and R3 are increased about 2-fold in IFN-treated HeLa extracts relatively to extracts from non-treated cells. R1 and R3 seem to involve the same DNA sequence in the probe since they react identically to competitors. Two proteins of 69 and 46 kDa form the IRS specific complexes as revealed by UV cross-linking. Identical DNA probes bearing either the hE-IRS or moE-IRS form complexes of different characteristics with nuclear proteins, suggesting that the two IRS variants are the targets of binding of different proteins or of different protein complexes.

INTRODUCTION

The development of the biological effects of all types of Interferons (IFNs) depends on their binding to target cells surface receptors (1) and thereafter on the induction or increased expression of a specific set of genes (2). Several of these genes are cloned, and the transcription of some of them was shown to be increased as early as a few minutes after IFN addition to the culture medium (3,4). The transcriptional induction by type I IFNs (α/β) in general, does not require *de novo* or continuous protein synthesis. A genomic DNA element responsible for the transcriptional induction by type I IFNs has been defined in several IFN-inducible genes (5-13). The IFN responsive sequences (IRS) of the human and mouse (2'-5') A synthetase genes behave as inducible enhancers and are equally able to render the thymidine kinase promoter inducible by IFN-β (5,6) and IFN-γ (unpublished). The IRS of the human (2'-5') A synthetase, mouse H_2D, or human ISG 54 genes form specific complexes with nuclear proteins *in vitro* (6,8,10,14) which are probably transacting factors responsible for the changes in transcriptional activities of the gene promoters in the presence of IFN.

In the present work, to characterize further the function of the IRS and its associated proteins, we have mutated the IRS and correlated its function as a transcriptional enhancer with its binding of nuclear proteins *in vitro* tested by gel shift assays (15). Two IRS-specific complexes R1 and R3 are formed with the human (2'-5') A synthetase AluI fragment (Alu fgt,-112/-74 on the gene in ref. 5) of which the faster migrating R3 is the more abundant. The formation of both complexes increases about 2-fold in extracts treated for 1 hour with IFN while a third complex R2 is not influenced by IFN and not displaced by cold IRS competitors. Protein synthesis seems required to maintain complex R3 but not complex R1, suggesting that the latter is involved in transcriptional induction. Using UV-cross linking, we found that the human IRS-specific complexes are formed with proteins of 46 and 69 kDa. Two 40 bp DNA fragments containing either the murine moE-IRS or the human hE-IRS and differing only at the five positions where the two IRS vary, were used as probes in gel shift assays. The IRS-specific complexes formed by the mo-E-IRS probe migrated differently than the hE-IRS complex. In addition, cross competition between the two IRS for factor binding *in vitro* was not as efficient as homologous competition. This difference between closely related IRS could explain how in some cell mutants only a subset of the IFN inducible genes is stimulated by IFNs (16).

MATERIALS AND METHODS

Construction of fusion genes and cell transfections

Complementary strands of synthetic oligonucleotides were annealed for 2 min at 88°C, 10 min at 65°C, 10 min at 37°C, and 5 min at room temperature, in 10 mM Tris-HCl pH 7.6, 0.1 mM EDTA, and 50 mM NaCl. The hE-IRS mutants were synthesized in a SalI linker in 5' to facilitate cloning in the multiple cloning site of the pGEM TK CAT-O vector cut with SphI (blunt-end) and SalI (6). hE-IRSmI is tcagTTAGGAAAGCAAACCAg and hE-IRSmII is tcagGAAACGACGAAACCAg. The hE-IRS, moE-IRS and mo-E-IRSm, similarly prepared, were cloned in the same way (6). The 2XhE-IRS TK CAT construct was built as the XhE-IRS (6) and contains two head to head hE-IRS in the BamHIII site of the pGEM TK CAT 0 polylinker (6).

Transfections in HeLa cells and CAT assays were done as before (5,6). Analysis of the RNA transcripts in the transfects cells by SI-nuclease assays was performed as described (6).

Preparation of nuclear extracts

Nuclear extracts of monolayer HeLa cells were prepared as Dignam et al. (17),

except that a mixture of protease inhibitors was added to all the buffers from the lysis step: leupeptine, 0.1 µg/ml, aproptinin 4 µg/ml, chymostatin 2 µg/ml, pepstatin 1.5 µg/ml, antipain 2 µg/ml. The proteins were extracted stepwise from the nuclear pellet, which was first suspended in 0.3 ml/10^8 cells of Dignan's buffer B (0.14 M NaCl) and supplemented with the same volume of buffer B (0.7 M NaCl). After gentle mixing at 0°C for 30 min and centrifugation, the 10,000xg supernatant was analyzed for 5 hours against 500 volumes of 20 mM Hepes , pH 7.9.

The end-labelled DNA (10,000 cpm, about 7 pmoles) was mixed with 2.5 µg poly dI-poly dC (Pharmacia) and incubated for 30 min at room temperature with 3µg of proteins in 60 mM KCl, 20 mM Tris-HCl pH 7.5, 0.2 mM EDTA, 5 mM DTT and 0.7 mM spermidine in 20 µl. The binding reactions were done without competitors or with 100 molar excess of cold competitors over the radiolabelled probe. Analysis of the DNA-protein complexes was done on 4% polyacrylamide gels in 0.5x TBE or 0.5x Tris-glycine buffers. All the cold dsDNA fragments used as competitors, either extracted from plasmids or chemically synthesized, were first treated with Klenow enzyme in the presence of 0.5 mM dXTP and purified by Phenol-Chloroform before ethanol precipitation.

DNA-proteins UV-cross-linking

The cross-linking was done essentially as described (19). The Alu fgt cloned in the pGEM3 vector was cut out by BamHI-HindIII and cloned in the Bluescript (M13-) vector (Stratagene). The T3 primer was used to prime the synthesis of the non-coding strand of the insert in the presence of 66 µM dATP, dGTP, 5-bromo 2'-deoxyuridine triphosphate (Pharmacia) and 6 µM α-^{32}P dCTP. The DNA was digested with HindIII and BamHI and purified by acrylamide gel electrophoresis. Nuclear extract (9 µg of proteins) was mixed with 10,000 cpm of probe and 5 µg of poly dI poly dC and incubated for 30 min at room temperature in Nunc tubes in the same conditions as above. The tubes, in iced water, were covered with Saran Wrap and UV irradiated for 20 min by a Mineralight lamp, 254 nm, placed 20 cm above. After adding $CaCl_2$ to 10 mM, the probes were digested for 30 min at 37°C with 2 µg of DNAseI (Worthington) and one unit of Micrococcal Nuclease (Pharmacia). Proteins were denatured by 5 min heating at 95°C in sample buffer and analyzed on 10% polyacrylamide/SDS gel. The gels were enhanced for fluorography, dried and autoradiographed.

RESULTS

Functions of IRS variants *in vitro*

In Table I, the IRS-like sequences of genes inducible by type I IFN (5-9,11) or type

TABLE 1

Variants of the interferon responsive sequence and consensus IRS

Gene	Reference	Sequence Box I	Position on the gene
Human 2'-5' A synthetase hE-IRS	(5)	G A G G A A A C G A A A C C	(-101/-88)
Mouse 2'-5' A synthetase moE-IRS	(6)	G G G A A A T G G A A A C T	(-72/-59)
Mouse H_2K^b	(11)	G C A G A A G T G A A A C T	(-138/-151)
Human 6-16 gene	(8)	G G G A A A A T G A A A C T	(-112/-99)
Human ISG 15	(7)	G G G A A A C C G A A A C T	(-108/-96)
Human ISG 54	(7)	G G G A A A G T G A A A C T	(-127/-140)
Mouse Mx protein	(9)	T C A G A A A C G A A A C T	(-118/-131)
Human Factor B	(20)	A G G A A A C A G A A A C T	(-127/-140)
Human IP_{10}	(21)	T G G A A A G T G A A A C C	(1913/1926)
CONSENSUS		G N PuPuA A N N G A A A C Py 1- 2- 3- 4 - 5- 6- 7- 8- 9- 0- 1- 2-3- 4	
hE-IRS mutant I	this work	* T A G G A A A C G A A A C C	
HE-IRS mutant II	" "	* * G A A A C G A C G A A A C C	
moE-IRS mutant	" "	* * * G G G A A A T G G C G T C G	

The most conserved positions are shown in the frame with the GAAAC box I. The nucleotides in the consensus IRS are numbered 1 to 14. In the mutants used in this work, the residues mutated relatively to the consensus are shown by asterisks.

II IFN (20,21), have been aligned on the very conserved box (GAAAC). Two AA residues, and a G residue, located respectively two and eight nucleotides upstream from this box I, are also very conserved in all the sequences, the G being present in most type I IFN (α/β) responsive genes. In hE-IRSmI, we mutated the first G into T and in hE-IRSmII we replaced the two A by CG. In hE-IRSmII, we conserved the duplication of the GAAA motif found in almost all the IRS (Table

TABLE II
Interferon-inducible enhancer activity of IRS variants

Plasmids pGEM TK CAT	Induction ratio
hE-IRS (-102/-87)	3.7
hE-IRS mutant I	4.0
hE-IRS mutant II	0.9
moE-IRS (-74/-59)	3.3
moE-IRS mutant	0.75
pGEM TK CAT-O	0.6

HeLa cells were transfected with the indicated plasmid DNA (5,6). About 24 hours after transfection, duplicate plates were either treated with IFN-β_1 (250 U/ml) or mock treated. After another 24 hours, CAT activity was measured in the cell extracts (20). The ratio of CAT activity in the presence of IFN to CAT activity in its absence is shown.

I), the repeats being now separated by 4 nucleotides instead of one or two in the native IRS variants. In moE-IRSm, the three A of box I were changed into CGT.

The function of the native and mutated IRS similarly cloned in the pGEM TK CAT-O vector was first tested by measuring the CAT activity in transfected HeLa cells treated not by IFN-β_1, for 24 h (Table II). By this test we have shown that the hE-IRS-mI is as efficient as the native hE-IRS to mediate the activation of transcription by IFN-β_1. The mutation of the hE-IRS-mII on the contrary completely suppressed the ability of IFN to induce the CAT gene and CAT activity was even decreased by IFN. As reported previously (6), mutation in the conserved box I abolished the transcriptional induction. To verify that these differences in CAT expression reflect transcriptional activities, we examined the RNA transcripts by S1 nuclease analysis with a uniformly labeled DNA probe (Fig. 1). The RNAs that start at the TK promoter protect the 309 bp f2 fragment, while transcripts initiated upstream correspond to the larger f1 fragment. The hE-IRSmI responds to IFN by an increase in RNA transcripts from the TK promoter, and also from upstream RNA starts. In contrast, hE-IRSmII gave non-inducible transcripts. Very low transcription was seen from the TK promoter with the PGEM TK CAT 0 vector.

With the active IRS, the CAT activity was inducible 3 to 4-fold by IFN. However, taking into account that the CAT activity from the vector itself is

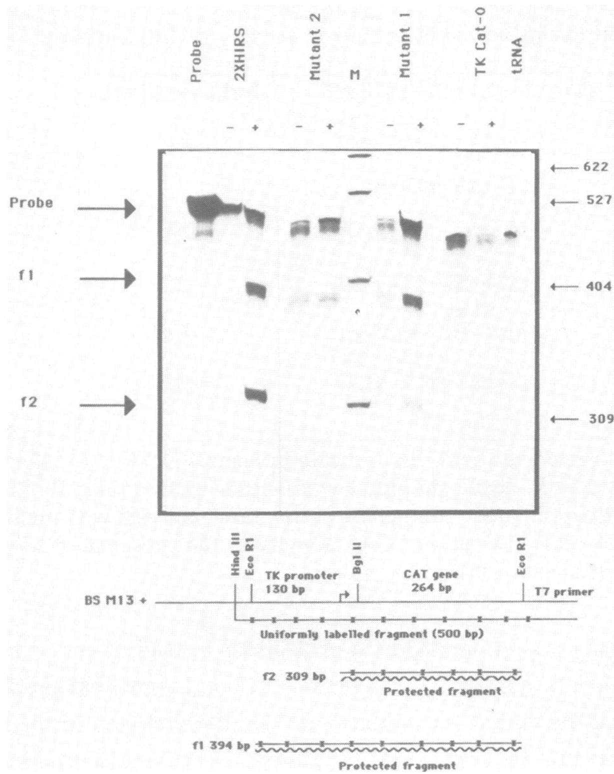
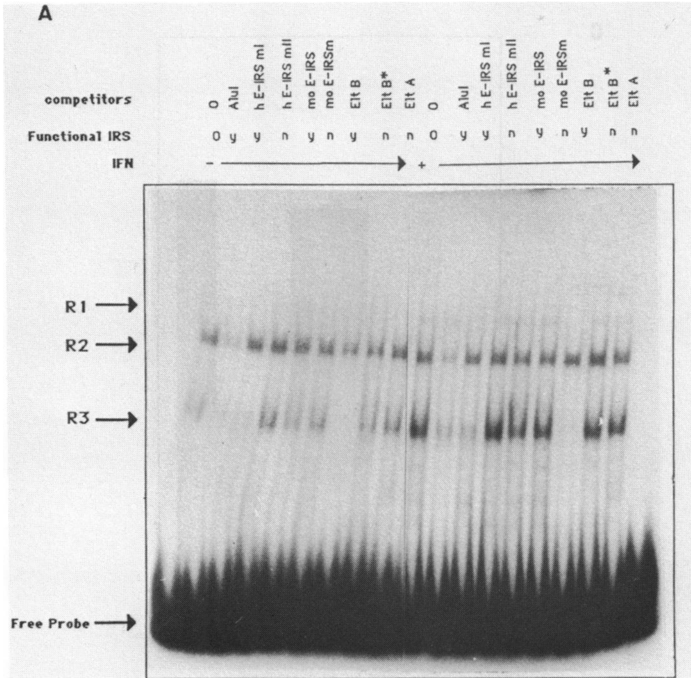


Figure 1.
Inducibility of transcription by IFN on the chimeric IRS-containing constructs. HeLa cells were transfected with the indicated plasmid DNAs, and treated 24 hours later with 250 U/ml of IFN-β_1. Cells were harvested 12 hours afterwards and RNAs extracted. The RNAs were hybridized to the single stranded probe uniformly labelled with α-^{32}P dCTP, generated as shown below the gel and as described (6). The fragments f_1 and f_2 protected by transcripts against S1 nuclease are schematized. f_2 corresponds to transcripts starting at the TK cap site (31). M = MspII-cut pBR322 molecular weight marker.

reduced 1.6 fold by IFN, the actual induction is at least 6-fold. Scanning of the signal of the S1-nuclease resistant fragments in Fig. 1 indicated even greater enhancement.

In vitro DNA-protein complexes specific of the Interferon-Responsive Sequences

Electrophoretic band shift assays (15) were used in an attempt to correlate the function of the IRS mutants to activate gene expression in transfected cells *in vivo* with their ability to bind nuclear protein factors *in vitro*. Figure 2 shows typical results obtained with the radiolabeled Alu fragment probe (Alu fgt, Fig 3A)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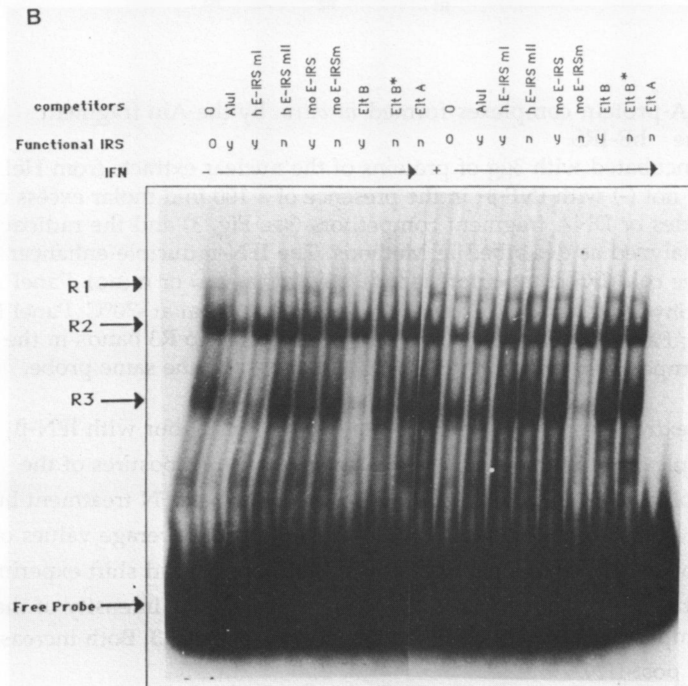
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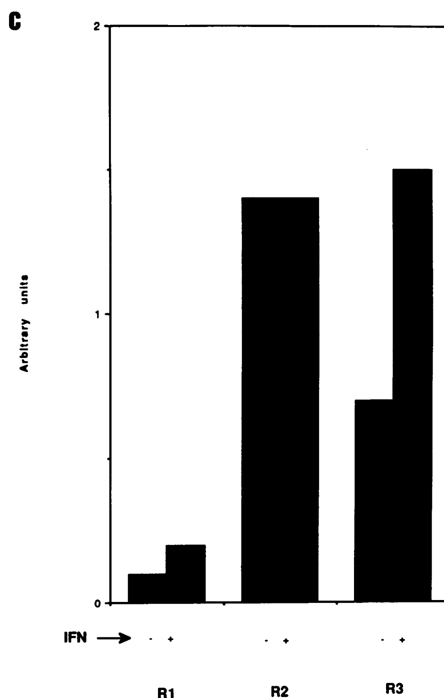

Figure 2.
Labelled DNA-protein complexes formed *in vitro* by the Alu fragment containing the hE-IRS.
The Alu fgt incubated with 3µg of proteins of the nuclear extracts from HeLa cells treated (+) or not (-) with INF-β_1 in the presence of a 100 fold molar excess of cold oligonucleotides or DNA fragment competitors (see Fig. 3) and the radioactive complexes analyzed as described in Methods. The IFN-inducible enhancer function of the cold IRS competitors is indicated by y (yes) or n (no). Panel A = autoradiography after 16 hours exposure to Agfa Curix film at -70°C. Panel B = 3 day exposure; Panel C = Summary of scanning of the R1 to R3 bands in the absence of competitors, using different HeLa extracts and the same probe.

with nuclear extracts from HeLa cells treated or not for 1 hour with IFN-β_1. Three retarded complexes R1, R2 and R3 are visible on the two exposures of the autoradiography (Fig. 2A,B). R1 and R3 are increased after IFN treatment but R2 did not show a significant change. Fig 2C summarizes the average values obtained by scanning overnight exposures from gels of 3 different band shift experiments with different nuclear extracts but with the same probe. The intensity of the slow migrating complex R1 is about 10 times lower than that of R3. Both increase about 2-fold 1 hour post-IFN.

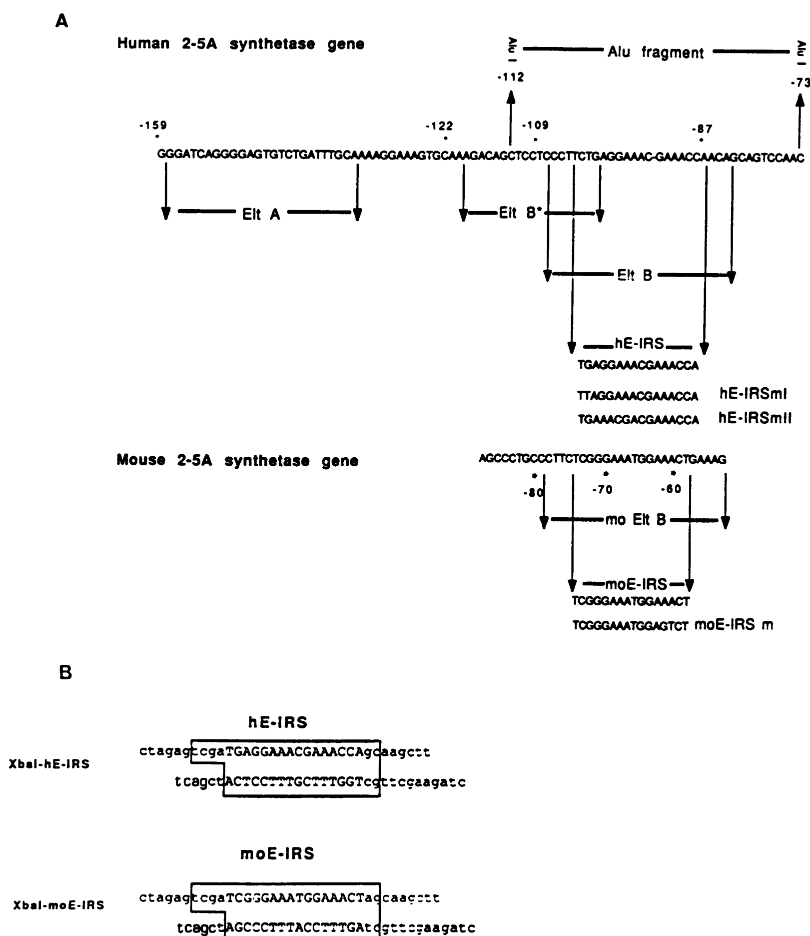

Figure 3.
DNA probes and competitors used in gel-shift assays.
Panel A = The human element A (Elt A), element B (Elt B), oligonucleotide B* (Elt B*), or mouse element B (moElt B) are synthetic blunt ended DNA fragments corresponding to the human (5) or mouse (6) (2'-5') A synthetase sequence. The hE-IRS, hE-IRSmI, hE-IRSmII, moE-IRS, moE-IRSm, are synthetic oligonucleotides which contain in addition to the shown sequence a SalI linker in 5' (see ref. 6 and Methods). They were all treated with the Klenow enzyme after strand reassociation and used as cold competitors. The Alu fragment was cloned in the polylinker of pGEM-3 and radiolabelled as described in Methods. Panel B = The XbaI fragments extracted from the hE-IRS TK CAT, or moE-IRS TK CAT plasmids (6) were labelled by Klenow as described in Methods.

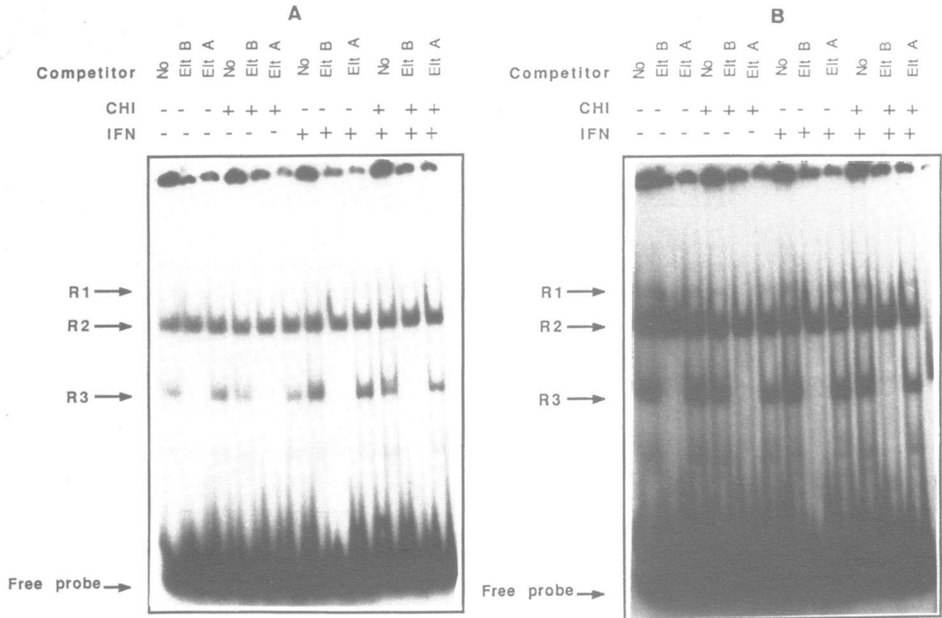

Figure 4.
Effect of cycloheximide treatment on DNA-protein complex formation.
In vitro DNA-protein binding was done with the Alu fgt probe and the complex analyzed as described in Methods. The extracts were prepared from cells treated (+) or not (-) with cyclohexmide (CHI) or IFN for 1 hour. Panel A = One overnight exposure. Panel B = 4 days exposure.

<u>Correlation between IFN-dependent enhancer activity and protein binding</u>

The formation of complex R3 is inhibited by the addition of 100-fold molar excess of the 25 bp element B (Elt B, Fig. 3A) from the human (2'-5') A synthetase gene, that contains the hE-IRS (Fig. 2). It is not inhibited by the same molar excess of the 25 bp element A (Elt A, Fig. 3A), which is part of the second enhancer of the human (2'-5') A synthetase, distinct from the IRS (5). Formation of the R2 complex is neither inhibited by Elt A nor by Elt B, but is prevented by the Alu fgt itself (Fig. 2). It, therefore, denotes the binding of a protein outside the IRS. Since the oligonucleotide B* (Fig. 3A) corresponding to the 5' end of the Alu fragment, is also unable to compete against R2 formation, R2 must result from binding of a protein either to the 3' end of the Alu fragment or to the short polylinker tails.

Figure 2 shows that hE-IRSmI which has an active IFN-dependent enhancer activity (Table II) competed against formation of complex R3, as well as the wild

type Elt B sequence and the complete Alu fragment. The hE-IRSmI oligonucleotide also reduced formation of R1 with IFN-treated nuclear extracts (see long exposure of Fig. 2B). In contrast, the inactive mutant hE-IRSMII, was clearly unable to compete against formation of the hE-IRS-protein complexes. Hence, its inability to respond to IFN in transfected cells is reflected in its inability to bind the proteins which specifically recognize the wild type IRS.

Effect of protein synthesis inhibitor on IRS-protein complex formation

Activation of transcription in response to IFN-β1 does not in general require ongoing protein synthesis (22-24) and is not inhibited by the addition of cycloheximide. If one of the IRS-protein complexes represents the binding of a protein which activates transcription of the gene, its formation in the presence of IFN should not be inhibited by cycloheximide. We, therefore, compared IRS-protein complex formation with nuclear extracts from HeLa cells treated with IFN in the presence and absence of cycloheximide, both for 1 hour. A reduction in the amount of R3 complex formed with the hE-IRS probe was noted when the cells had been treated by cycloheximide in the presence or absence of IFN (Fig. 4). The reduction was seen more clearly when the complex was formed in the presence of the non-specific competitor Elt A. Under the same conditions, the R1 complex seemed unchanged but the low yield of this complex prevents more precise quantitation in this experiment. Complex R2 was always unaffected by either IFN or cycloheximide.

Molecular weights of the hE-IRS-binding proteins

Having characterized the IRS-specific complexes, we used the approach developed by Chodosh et al. (19) to determine the molecular weights of the proteins binding to the Alu fragments. The fragment was cloned in the Bluescript vector (see Methods) and the non-coding strand of the insert was synthesized using 5-bromo 2' deoxyuridine (5BrdU) instead of thymidine, to favour cross-linking, and labeled by α-^{32}P dCTP. This did not affect the formation of specific complexes in HeLa extracts (Fig. 5A and B). Increased binding after IFN was clearly observed. After UV-irradiation, the DNA was degraded by nucleases and the proteins analyzed in denaturing conditions (Fig. 5C). Three radioactive bands are visible on the autoradiography. A strong signal appears at the level of a 46 kDa protein, a weak one at the level of a 69 kDa protein (Fig. 5C). Both are decreased or not detectable where the Elt B (containing the hE-IRS) was added as competitor, and not changed where Elt A was used (Fig. 5B). By contrast the 100 kDa band was not inhibited by either competitor. The 46 kDa and 69 kDa proteins are thus good candidates for being the transacting factors that bind to the IRS. When DNAseI was added before UV-treatment, no such signals were generated (not shown).

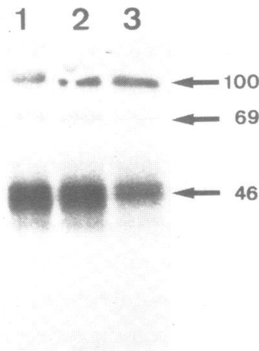

Figure 5.
UV cross-linking of IRS-binding proteins.
The Alu fragment was cloned in the Bluescript vector and the non-coding strand uniformly labelled by 5BrdUTP and $\alpha^{32}P$ dCTP (see Methods). The ds-DNA probe excised from the vector was mixed with HeLa nuclear extracts treated or not by IFN and used for gel shift assays (Panels A,B), or UV cross-linking and analysis on SDS-polyacrylamide gel (Panel C). The molecular weights were determined by references to radiolabelled protein markers (Amersham) run in a parallel lane.

When the coding strand was labeled, no 5BrdU could be incorporated in the IRS (Fig. 3) and neither the 46 kDa nor 69 kDa protein were labeled providing further evidence that these proteins are bound to the IRS.

Human nuclear proteins form different complexes with the murine and human E-IRS

A surprising result was that under the present experimental conditions, the mouse E-IRS oligonucleotide functionally equivalent to the hE-IRS (Table II) did not compete efficiently against protein binding to the hE-IRS (Fig. 2). At a 100-fold molar excess, the moE-IRS did not compete against hE-IRS more than 30-50% as judged by gel scanning. Formation of the R3 complex with the human E-IRS could be eliminated by the murine -E-IRS, as previously reported (6), but only at higher molar ratios. To examine the basis for this specificity, we repeated the protein binding experiments with the moE-IRS as probe. A moE-IRS probe was excised from the pGEM moE-IRS TK CAT plasmid (6) with XbaI and labeled by filling-in with Klenow enzyme and α-^{32}P dCTP. A similar probe was prepared from the human counterpart pGEM hE-IRS TK CAT (6) as shown in Fig. 3B. Though these two fragments differ only in their respective IRS, their band pattern in gel retardation is different (Fig. 6A and B). The XbaI hE-IRS (Fig. 6A) forms a major complex R3' of mobility similar to the major complex R3 formed

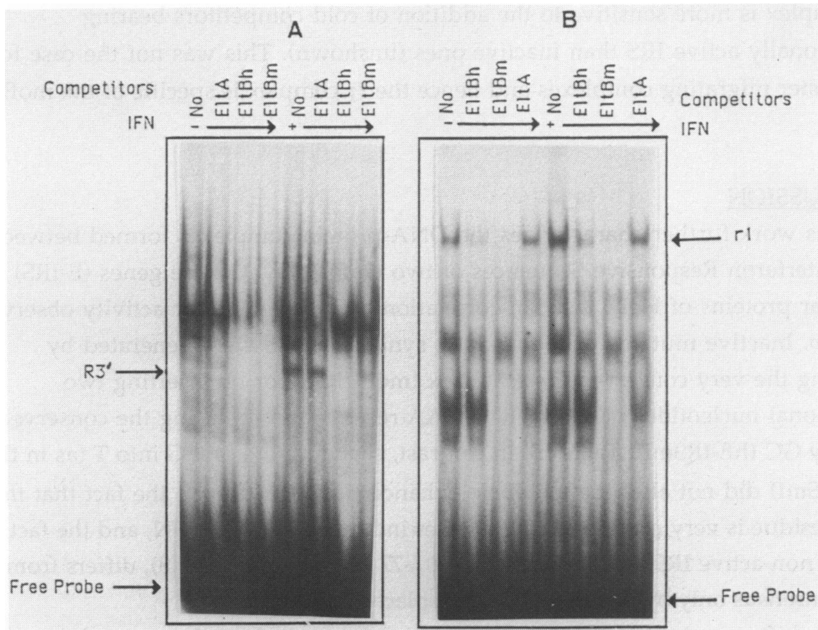

Figure 6.
Electrophoretic migration of IRS-protein complexes formed by the hE-IRS and moE-IRS. Autoradiography (overnight exposure) of gel shift assays with different DNA probes using the same IFN treated (+) or non-treated(-) HeLa extracts. Panel A: The probe used was hE-IRS XbaI (see Fig. 3B); Panel B: The probe was the moE-IRS XbaI (see Fig. 3B).

by the Alu fragment (Figs. 2 and 4). Some increase of the R3' band intensity like R3 intensity is observed in IFN-treated extracts. Both R3 and R3' formation can be completely inhibited by the homologous Elt B, less well inhibited by the oligonucleotide carrying the moE-IRS sequence, and is not inhibited by the Elt A (Fig. 6 A). This strongly supports the interpretation that the two fragments, which have only the hE-IRS in common, bind the same protein to form the R3' or R3 complexes with the IRS. The R1 complex is not visible after one overnight exposure to film and is indeed very weak also when the Alu fgt is the probe used.

Among the complexes formed by XbaI moE-IRS (see Fig. 3B), the complex r_1 is the only one whose intensity increases using IFN-treated extract (Fig. 6B). The oligonucleotides bearing the moE-IRS displaces the r_1 complex completely (Fig. 6B); the human IRS Elt B is less efficient than oligonucleotides bearing the moE-IRS, but more efficient than Elt A for competition (Fig. 6B). More generally, the

r1complex is more sensitive to the addition of cold competitors bearing functionally active IRS than inactive ones (unshown). This was not the case for the faster migrating complexes and hence the r_1 complex is specific of the moE-IRS.

DISCUSSION

This work further characterizes the DNA-protein complexes formed between the Interferon Responsive Sequences of two (2'-5') A synthetase genes (E-IRS) and nuclear proteins of HeLa cells, in correlation with the enhancer activity observed *in vivo*. Inactive mutants of the (2'-5') A synthetase IRS were generated by altering the very conserved GAAAC box (moE-IRSm) or by inserting two additional nucleotides between the GAAA repeats and replacing the conserved AA by GC (hE-IRSmII, Table II). In contrast, changing the first G into T (as in the hE-IRSmI) did not alter the inducible enhancer function despite the fact that this first residue is very conserved in the genes inducible by type I IFN, and the fact that a non-active IRS-like sequence, at -90/-77 of the 6-216 gene (8), differs from the consensus only by this first G being replaced by a C.

Gel shift experiments showed a correlation between the enhancer activity of the various E-IRS in transfected HeLa cells, and their ability to form specific complexes with HeLa nuclear proteins (see Fig. 2). The two specific complexes R1 and R3 formed with the Alu fragment containing the IRS, involve the same DNA sequence since both were affected in the same way by the various mutant competitors. The importance of the properly spaced AA at positions 5,6 versus the GAAAC box I is demonstrated by the fact that mutants with either no GAAAC (moE-IRSm) or with the AA separated by four nucleotides from box I (hE-IRSmII) were no more able to displace R1 as well as R3. Before and after IFN treatment, R1 and R3 have similar properties and the increase in DNA binding could result from a post-translational modification of the same proteins as suggested for other inducible enhancers (25-27). This increase (1.5-2-fold) may account for only part of the transcriptional induction (about 6-fold). Possibly, the transcriptional activation domain of the IRS-binding proteins is modified by IFN treatment leading to full activation of transcription as suggested (25-27).

In several previous studies it was observed that complete inhibition of protein synthesis during cell treatment by type I IFN does not abolish the transcriptional activation of the inducible genes (21-23). On the other hand, the down-regulation of transcription which occurs a few hours post-treatment could be prevented by inhibitors of protein synthesis (23). After one hour of cycloheximide treatment, under conditions where 95% of the protein synthesis was abolished, we observed

a decrease in the R3 complex in the presence or absence of IFN whereas the amount of R1 complex did not appear to change (see Fig. 4). This could support a model in which the R1 complex is the one responsible for the transcriptional activation. However, it is not evident that R3 corresponds in turn to the putative repressor complex causing down-regulation, since it increases as early as one hour post-IFN induction when transcription is still maximum (4). The experiments with cycloheximide must be viewed with caution since inhibition of transcriptional activation by IFN in HeLa cells was also reported (24,28,29) and differences between various IFN-inducible genes were observed in the same cells (28,29). The differential sensitivity of the R1 and R3 complexes to cycloheximide could reflect the fact that the proteins involved in the formation of these two complexes have different turnover rates.

The involvement of different proteins in the R1, R2 and R3 complexes is further supported by the detection of three proteins of 46,69 and 100 kDa which are labeled by cross-linking to the Alu probes. The 100 kDa protein seems to correspond to complex R2 which is not competed for by elements B or A. Only the two other proteins, 46 and 69 kDa, appear specific for the IRS since their presence is decreased by addition as competitor of element B (containing the hE-IRS) but not of element A lacking the E-IRS. Further evidence of the specificity of these two cross-linked proteins is that probes in which the IRS was not substituted by 5-BrdU were not covalently bound to these proteins while generating the usual pattern in the gel shift assay. That the 46 kDa protein is more strongly labeled than the 69 kDa protein suggests that the former represents the more abundant R3 complex and the latter the less abundant R1 complex. We assume that both the 46 and 69 kDa proteins bind to the E-IRS enhancer. Other enhancer elements also bind two proteins, as for example the immunoglobulin gene octamer (30). Whether these different proteins carry out different functions such as activation and repression of transcription will have to be tested by *in vitro* transcription assays.

Two IRS-specific complexes were found associated with the IRS of the human 2'-5' A synthetase in COS-7 cells (10) and in Daudi cells (6) Using DNA probes containing the IRS region of the ISG 54 gene (14) or H_2L^d gene (12), with HeLa cell extracts (14) or T-cell extracts (12), complexes with similar characteristics to R1 and R3 were found. Complex formation was clearly increased after IFN treatment (12,14) but the proteins were not identified. Our results indicate that there may be more than one set of two proteins binding to various homologous IRS enhancers for different genes. Indeed, the mouse E-IRS was found not to compete well for the formation of protein complexes with the human E-IRS and when used as a

probe the mouse E-IRS formed complexes which differed from those formed with human E-IRS. Conversely, hE-IRS did not compete as well for the formation of the moE-IRS complex as the homologous sequence. This result is surprising in view of the very similar enhancer activity in human HeLa cells of the two E-IRS elements when cloned in front of the TK CAT gene. It was however reproduced with extracts from several other cells. The two homologous IRS could be recognized by different human proteins or alternatively the difference could reflect dimerization or conformational changes upon binding to the moE-IRS. Factor purification, to reduce non-specific binding on the IRS XbaI probes, and cross-linking experiments are in progress to answer this question. The apparent difference between protein complexes on two comparable IRS may be related to the observation that some cell variants can induce only a subset of the genes normally activated by IFN (16). There may be conditions in which slightly different types of IRS enhancers have different *in vivo* activities.

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