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Cells resistant to interferon are defective in activation of a promoter-binding factor

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Human cultured cell lines deficient in their ability to respond to type I interferon (IFN) fail to interrupt cellular proliferation or to induce an antiviral state following exposure to IFNα. Comparison of non-responsive Daudi and HeLa cell lines with IFN-responsive partner cell lines and examination of non-responsive Raji cells showed that the defective cell lines expressed type I IFN receptors of typical number and affinity and bound IFN equivalently compared to the normal cells. However, transcriptional induction of interferon-stimulated genes (ISGs) was greatly reduced and delayed in these cell lines, leading to reduced accumulation of ISG mRNA. Furthermore, the rapid activation of IFN-stimulated promoter binding factors whose appearance correlates with ISG transcriptional induction, did not occur in non-responsive cells Thus, the primary defect of these cells leading to an impaired physiological response to IFN appears to be an inability to activate promoter-binding factors necessary to trigger ISG transcription, an obligate early step in antiviral and antiproliferative physiology.

Key words: transcriptional control/nuclear factors/interferon stimulated genes

Introduction

Binding of type I interferon (α or β-IFN) to its cell surface receptor elicits several physiological responses in cultured cells, including inhibition of cell proliferation and inhibition of viral replication (Lengyel, 1982; Tamm *et al.*, 1987). These ultimate effects of IFN, detectable after 24 h, are preceded by a transient transcriptional activation of a group of interferon-stimulated genes (ISGs) whose protein products seem likely to be involved in physiological responses to IFN (Larner *et al.*, 1984, 1986; Friedman *et al.*, 1984; Kelly *et al.*, 1984; Levy *et al.*, 1986; Kusari and Sen, 1987; Reich *et al.*, 1987). Transcriptional activation of ISGs is associated with the rapid induction of DNA-binding proteins (Levy *et al.*, 1988; Porter *et al.*, 1988; Rutherford *et al.*, 1988; Cohen *et al.*, 1988) that bind to the interferon stimulation response element (ISRE), an ISG regulatory element that is both necessary (Levy *et al.*, 1986, 1988; Reich *et al.*, 1987; Porter *et al.*, 1988; Rutherford *et al.*, 1988; Cohen *et al.*, 1988) and sufficient (N.Reich and J.E.Darnell,Jr, unpublished; Cohen *et al.*, 1988) for interferon stimulation. One IFN-induced DNA-binding activity, designated B3 (Levy *et al.*, 1988), is implicated in transcriptional activation because its accumulation directly parallels transcriptional activation. As is the case for transcriptional activation of ISGs, the production of this activity in response to IFN treatment is rapid, does not require new protein synthesis, and probably results from an IFN-induced post-translational modification of an inactive precursor (Levy *et al.*, 1988). In this report we show that three human cell lines (Daudi, Raji, and HeLa lines) are resistant to the physiological effects of IFN even though they have normal numbers of high affinity IFNα receptors. IFN-induced transcriptional activation of three ISGs (ISG15, ISG54, and ISG56) was found to be greatly reduced in the IFN-resistant lines as compared with sensitive lines. In addition, the IFN induction of the B3 DNA-binding activity was very low or undetectable in all IFN-resistant lines studied. These results indicate that the IFN-resistant lines are defective in the activation of a promoter-binding factor, an early event in the IFN response, and thus fail to transcribe a set of genes necessary for the physiological response.

Results and discussion

Interferon-resistant cell lines have normal interferon receptors

The IFN-sensitivity of human tissue culture cells [two HeLa cell lines, two subcloned Daudi cell lines (Dron and Tovey, 1983), and the Raji cell line] was determined by quantitation of the antiproliferative effect of IFNα on these lines as well as by determination of the resulting antiviral state. IFNα treatment significantly inhibited proliferation of sensitive Daudi cells and one HeLa cell line, while proliferation of the Raji and variant Daudi cell lines was unaffected by IFNα treatment, and the proliferation of a second HeLa line was only slightly impaired (Table I). In addition, vesicular stomatitis virus (VSV) infection of sensitive HeLa or Daudi cells was largely blocked by IFNα treatment, while treatment of the variant HeLa or Daudi line was ineffective (Table I).

A trivial explanation of the IFN-resistance of these cell lines would be the possible absence of IFN receptors. The presence of type I IFN receptors was determined by the binding of [^{125}I]IFNα to IFN-sensitive and resistant cell lines. Scatchard analysis of IFN binding data revealed that the resistant and sensitive lines expressed similar numbers (1000–4000 receptors/cell) of high affinity (K_d ~50–100 pM) cell surface receptors for IFNα (Table I). Some differences between lymphoblastoid and HeLa cell lines were observed, but these are not relevant to differences between IFN-sensitive and resistant cells of each type.

Transcription of ISGs in IFN-resistant cells

Subcloned IFN-resistant Daudi cell lines have been studied by several laboratories (Dron and Tovey, 1983; Silverman *et al.*, 1982; Hanigan *et al.*, 1984; Dron *et al.*, 1985; McMahon *et al.*, 1986). Neither the increase in mRNA

Table I. The number of high affinity IFNα receptors, the affinity of the receptor for IFNα, and the effects of IFNα on cell proliferation and virus replication

Cell line	Number of IFNα receptors/cell	Affinity of IFNα binding (K_d), pM	Doubling time (h)		Log reduction in virus replication
			+IFN	Control	
HeLa-sen	1200	100	29	18	3.5
HeLa-res	1100	120	19	18	1.1
Daudi-sen	3700	50	45	24	4.0
Daudi-res	3000	45	24	23	0.3
Raji	1900	60	23	23	0.4

sen = IFN-sensitive, res = IFN-resistant.

encoded by a group of IFN-induced genes [561 = ISG56 (Tiwari *et al.*, 1987), 1−8, 6−16 and 9−27 (McMahon *et al.*, 1986)], at least three of which (561, 6−16, and 1−8) are known to be transcriptionally induced, nor the IFN-induced post-transcriptional decrease of c-*myc* (Jonak and Knight, 1984; Knight *et al.*, 1985; Dron *et al.*, 1986) and μ-chain (Meurs and Hovanessian, 1988) mRNAs occurs in resistant Daudi cells treated with IFNα. Some mRNAs that are known to increase after IFN treatment of sensitive cells (thymosin β4, metallothionein II, HLA2A, and 2′-5′ oligo-A synthetase) also increased in resistant cells (McMahon *et al.*, 1986). In no case was the level of regulation for any of these mRNAs determined in the IFN-resistant cell lines.

We therefore measured the IFN-induced transcription of ISGs in IFN-sensitive and resistant Daudi and HeLa cells in response to IFN by *in vitro* nuclear run-on transcription assays (Figure 1 and Table II). A 2-h IFNα treatment resulted in strong transcriptional signals for ISG15, ISG54 and ISG56 in sensitive Daudi and HeLa cells compared to a very low basal transcription rate. In contrast, resistant lines showed a very weak transcriptional response of these genes. There was much less increase in ISG54 or ISG56 transcription and ISG15 transcription was about one-third that found in sensitive cells. The basal levels of transcription for the three genes were somewhat higher in the resistant HeLa cells than in the sensitive HeLa cells. In resistant Daudi cells IFN did induce transcription of these genes, but only ~40% as well as in sensitive Daudi cells. As shown previously for fibroblasts (Larner *et al.*, 1986), transcription of ISG15, ISG54 and ISG56 in the sensitive HeLa line had declined 6 h after IFN addition, with a more rapid decline for ISG54 and ISG56 than for ISG15 (Reich *et al.*, 1988, and data not shown). The transcription rate of the 2′-5′ oligo-A synthetase gene (Rutherford *et al.*, 1988) was also tested in several experiments. For this gene the transcription signals after interferon treatment were considerably weaker than for the other genes. However, the IFN sensitive HeLa cells were at least 5-fold more responsive than the resistant lines. The Daudi sensitive cells were only about two times more responsive and again the transcription signals were low. The reports that this gene is normally expressed in the IFN-resistant Daudi line (McMahon *et al.*, 1986; Dron *et al.*, 1986), based on mRNA accumulation, might suggest a difference in cell lines or possibly that post-transcriptional control of this mRNA is also important in Daudi cells.

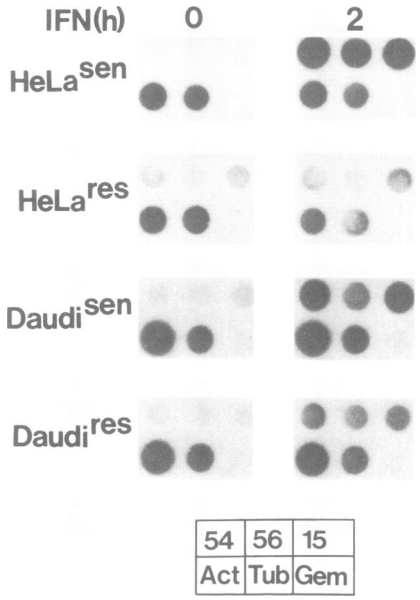

Fig. 1. Transcriptional analysis of ISGs in IFN-sensitive and resistant HeLa and Daudi cell lines. *In vitro* nuclear run-on transcription assays were performed with nuclei from untreated cells (**left panels**) or cells treated with IFNα for 2 h (**right panels**). Radiolabeled nascent RNA was hybridized to samples of ISG54, ISG56, ISG15, β actin, tubulin, and pGEM1 DNAs affixed to nitrocellulose. The pattern of DNA dots on each filter is diagrammed below. Sen = IFN-sensitive and Res = IFN-resistant.

Table II. Quantitation of IFNα-induced transcription[a]

Cell line	Gene	ISG15		ISG54		ISG56		OAS	
	IFN (h)	0	2	0	2	0	2	0	2
HeLa-sen		0	135	0	322	0	125	0	20
HeLa-res		6	47	2	13	2	4	0	4
Daudi-sen		2	51	0	57	0	15	0	6
Daudi-res		0	20	0	20	0	13	0	3

sen = IFN-sensitive, res = IFN-resistant.
[a]Run-on transcription assays were quantitated by densitometry of preflashed X-ray film. Several exposures were obtained to insure that signals were linear. Results are normalized to the signal from actin for each time point and presented as arbitrary units.

mRNA accumulation by IFN-sensitive and resistant cells

The accumulation of ISG mRNA for ISG15, ISG54, and ISG56 generally paralleled the transcription rate in all cell lines (Figure 2). In addition, very little ISG mRNA accumulated in IFN-treated Raji cells (data not shown). The presence of high levels of ISG mRNAs at 12 h in sensitive Daudi cells reflects the maintenance of high transcription rates, even 20 h after IFN addition (R.P. unpublished results), which appears to be a peculiarity of this cell line

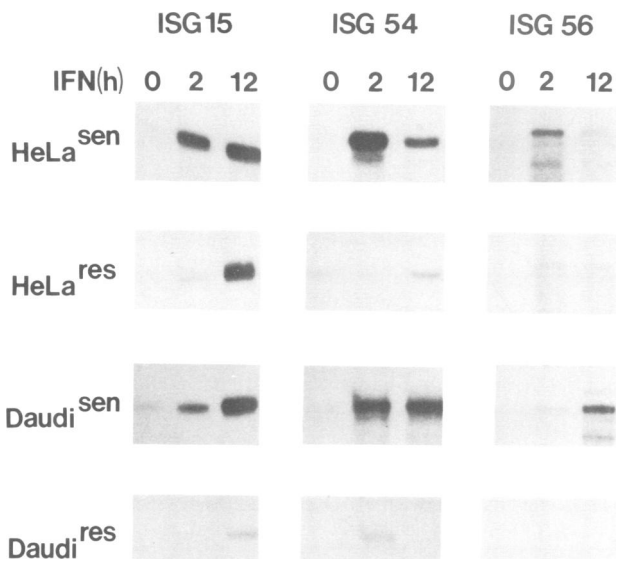

Fig. 2. ISG mRNA accumulation. Total cellular RNA was isolated from untreated cells or cells treated for 2 or 12 h with IFNα. The RNA from each sample was assayed by nuclease protection with cRNA probes for ISG15, ISG54, and ISG56. A probe for γ-actin (Enoch *et al.*, 1986) was used to demonstrate that equivalent amounts of RNA for each sample were assayed (data not shown).

(Tiwari *et al.*, 1987). An exception is the maintenance of a high level of ISG15 mRNA even after 12 h of IFN treatment in both sensitive and resistant HeLa lines. Given the low transcription rate of ISG15 after 12 h of IFN treatment (data not shown), these results imply that ISG15 mRNA is more stable in HeLa cells than the other ISG mRNAs.

DNA binding proteins in IFN-sensitive and resistant cells

We have recently described DNA-binding activities in human cells (HeLa and fibroblasts) that bind to the ISRE (Levy *et al.*, 1988). Nuclear extracts from treated or untreated cells contain one activity, designated B1. IFN-treated cells contain two additional DNA-binding activities, B2 and B3. The level of B3 activity increases rapidly in IFN-treated cells, even in the presence of cycloheximide, as does ISG transcription. Therefore, the B3 factor is a candidate for a positive activator of transcription. The B2 activity is produced only after 90 min of treatment and requires protein synthesis. Since protein synthesis is required for transcriptional repression of the ISGs, which occurs 2–8 h after IFN treatment, the B2 factor could be involved in transcriptional inhibition.

To detect any differences in the production of these DNA-binding activities in IFN-sensitive and resistant cells, extracts prepared 2 h after IFN treatment were incubated with a radiolabeled DNA fragment containing the ISRE and assayed by gel retardation (Fried and Crothers, 1981) (Figure 3). While IFN-treated sensitive HeLa (lane 2) and Daudi lines (lane 6) showed clear induction of the B2 and B3 activities, IFN-treated resistant HeLa cells produced only B2 activity (lane 4). Extracts of resistant Daudi (lane 8) and Raji (lane 10) cells contained a very low induced level of B3 activity (only detectable by over-exposure of the autoradiograph shown in Figure 3), although a constant low level of B2 activity was present with or without IFN treatment. We

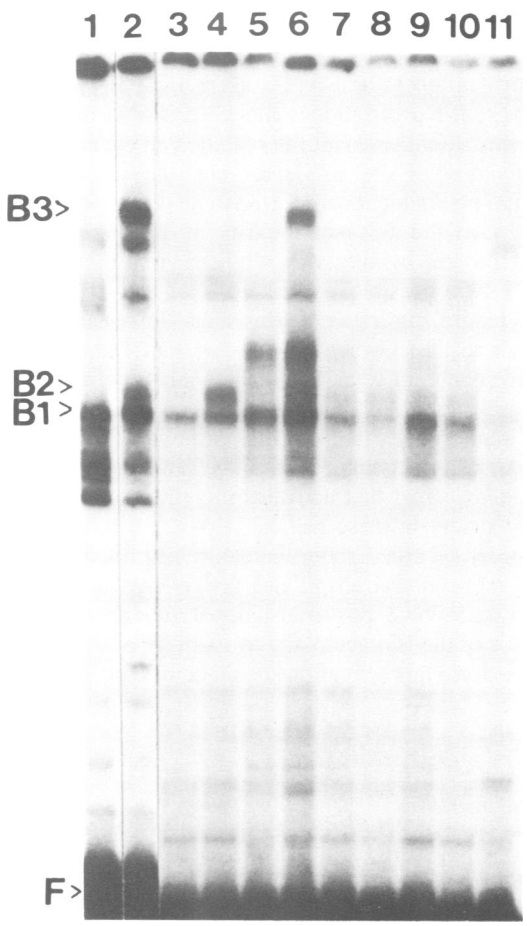

Fig. 3. Gel retardation analysis of IFN-induced nuclear DNA-binding proteins reveals the absence of the B3 complex in IFN-resistant cell lines. Nuclear extracts of untreated cells (**lanes 1, 3, 5, 7, 9**) or cells treated for two hours with IFNα (**lanes 2, 4, 6, 8, 10**) were incubated with the −115/−39 ISG15 probe (N.Reich unpublished) to form specific complexes. Extracts of IFN-sensitive HeLa (lanes 1 and 2) and Daudi (lanes 5 and 6) show IFN-dependent production of the B2 and B3 complexes. IFN-resistant HeLa cells (lanes 3 and 4) produce only the B2 complex. IFN-resistant Daudi (lanes 7 and 8) and Raji (lanes 9 and 10) lines produce a low level of the B2 complex regardless of treatment and overexposure of this experiment reveals a very low but detectable induced level of the B3 complex. The B1 complex forms with extracts of both treated and untreated cells. The faster migrating bands present in lanes 1 and 2 are due to partial degradation of the B1 complex and is variable between extracts. The complex migrating between the B3 and B2 complexes in lanes 5–10 is a slower mobility form of the B1 complex present in extracts of the lymphoblastoid lines. The specificity of the three indicated complexes for the ISG15 fragment is demonstrated in lane 11 in which the addition of 60-fold molar excess of unlabeled ISG15 sequences abolished the formation of detectable complexes from an IFN-sensitive HeLa extract. F = free probe.

emphasize that the level of B3 activity was very low or undetectable in extracts of all IFN-resistant lines studied.

The experiments presented here suggest that the critical defect in the IFN-resistant cell lines studied is the failure to activate an ISRE-binding factor in response to IFN treatment and that this factor, the B3 protein, is required for IFN-induced activation of ISG transcription. Furthermore, the action of the proteins whose synthesis is stimulated by the early burst of IFN-induced transcription must be responsible for the antiviral state and for causing cessation of cell growth.

As to the nature of the defect in the resistant lines it seems clear it is not an absence of IFN receptors; these were present in normal numbers and had a normal affinity for IFNα. Moreover, IFNα did induce the B2 factor in the resistant HeLa cells. In all resistant lines studied an event leading to the activation of the B3 factor and the associated ISG transcriptional induction appeared to be defective. Since the precursor to the B3 factor exists in IFN-sensitive cells (i.e. no protein synthesis is required to produce the B3 activity) two possible explanations of the IFN-resistant phenotype are suggested: (i) resistant HeLa cells have no precursor to the B3 factor while resistant Daudi and Raji cells have some (a weak transcriptional response was seen in these resistant lines), but not enough to elicit the late effects of IFN (antiproliferation and the antiviral state); or (ii) the B3 precursor is present in resistant cells but the IFN-induced intracellular signal that activates this precursor is reduced or non-existent in these cells.

As more is learned about the nature of proteins involved in IFN-induced transcriptional activation, an event that appears to involve post-translational modification of the precursor of the B3 factor, the availability of cell lines that fail to perform this activation should be of considerable use.

Materials and methods

Cells and interferon

Interferon responsive HeLa S3 cells, obtained from ATCC, and non-responsive variant HeLa cells, a gift from Dr E.Knight (DuPont), were maintained in monolayer cultures in DME supplemented with 10% fetal bovine serum. IFN-responsive and -non-responsive Daudi cells were obtained from Dr A.Hovanessian *et al.* (1986) and were cultured as static suspensions in RPMI-1640 supplemented with 10% fetal bovine serum. Homogeneous preparations of recombinant IFN-αA were generously provided by Dr P.Sorter (Hoffman-LaRoche) and added to media for HeLa cells at a concentration of 15 pM (500 U/ml) and for Daudi and Raji cells at 0.75 pM (25 U/ml).

Assay of IFN binding

Aliquots of cells were incubated at 3×10^6 cells/ml at 14°C with [^{125}I]IFNα-con1 (Alton *et al.*, 1983) at varying concentrations (3–100 pM) for 100 min. Cell-associated radioactivity was determined after centrifugation through a mixture of phthalate oils (Pfeffer *et al.*, 1987). Specific binding was determined as the difference between [^{125}I]IFNα bound in the absence and presence of unlabeled IFNα (5 nM). The data were plotted according to the method of Scatchard (1949) and analyzed by a non-reiterative least squares curve fitting program. The slope of plotted data yielded affinity of binding (K_d) and the *y*-intercept (bound ligand) was used to determine number of IFNα receptors/cell.

Antiproliferation assay

HeLa cells were plated at 1×10^5 cells per 25 cm^2 flask, treated for 3 days with IFNα at 15 pM (HeLa cells), and counted. Lymphoblastoid cell lines were grown in stationary suspension culture at a concentration of 2×10^5 cells/ml of medium and treated for 3 days with IFNα at 0.75 pM and counted. The ratio of cell number on day 3 to that on day 0 was used to calculate mean doubling times.

Antiviral assay

Cultured HeLa cells in 60 mm Petri dishes were incubated overnight with 15 pM IFNα, washed twice with PBS, and incubated with serial 10-fold dilutions of vesicular stomatitis virus (VSV-Indiana strain) for 1 h at 37°C. Cell monolayers were washed with PBS, overlayed with 0.9% agar, and virus plaques were counted 24–30 h post-infection. Viral titer on control HeLa cells was $1-3 \times 10^6$ plaque forming units/60 mm dish. Lymphoblastoid cells were incubated overnight with 0.75 pM IFNα and then infected with VSV at m.o.i. of 0.1 PFU/cell. Twenty-four hours later virus yield was assayed in L cells. Viral titer in infected control lymphoblastoid cells varied from 0.6 to 2.0×10^6 p.f.u./ml of medium.

RNA transcription and accumulation assays

Nuclear run-on transcription was performed as described (Larner *et al.*, 1984). $3-5 \times 10^7$ nuclei were used in each reaction and $5-20 \times 10^6$ c.p.m. of labeled nuclear RNA was hybridized to excess plasmid DNA bound to nitrocellulose.

RNA accumulation was measured with radiolabeled antisense RNA probes hybridized with 10 μg of total cellular RNA by RNase T_2 resistance (Levy *et al.*, 1986). The ISG15 probe was a 180 bp *Taq*I second exon fragment; the ISG54 probe a 250 bp *Eco*RI–*Taq*I second exon fragment; and the ISG56 probe a 400 bp *Eco*RI–*Bgl*II cDNA fragment (Levy *et al.*, 1988; Reich *et al.*, 1987).

DNA–protein binding assay

Nuclear extracts were prepared essentially as described by Dignam *et al.* (1983). Gel retardation analysis was performed essentially as described by Fried and Crothers (1981). End-labeled ISG15 fragment (−115 to −39) was incubated with 5–10 μg of nuclear protein in 40 mM KCl, 20 mM Hepes (pH 7.9), 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 320 μg/ml poly(dIdC):poly(dIdC), 40 μg/ml of a mutated ISG54 ISRE as non-specific competitor (M158 in Levy *et al.*, 1988), and 4% Ficoll in a final volume of 12.5 μl for 20 min. Protein–DNA complexes were separated from free probe on 4.8% polyacrylamide gels as described (Levy *et al.*, 1988).

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