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Complementation of a mutant cell line: central role of the 91 kDa polypeptide of ISGF3 in the interferon-α and -γ signal transduction pathways

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Mutants in complementation group U3, completely defective in the response of all genes tested to interferons (IFNs) α and γ, do not express the 91 and 84 kDa polypeptide components of interferon-stimulated gene factor 3 (ISGF3), a transcription factor known to play a primary role in the IFN-α response pathway. The 91 and 84 kDa polypeptides are products of a single gene. They result from differential splicing and differ only in a 38 amino acid extension at the C-terminus of the 91 kDa polypeptide. Complementation of U3 mutants with cDNA constructs expressing the 91 kDa product at levels comparable to those observed in induced wild-type cells completely restored the response to both IFN-α and -γ and the ability to form ISGF3. Complementation with the 84 kDa component similarly restored the ability to form ISGF3 and, albeit to a lower level, the IFN-α response of all genes tested so far. It failed, however, to restore the IFN-γ response of any gene analysed. The precise nature of the DNA motifs and combination of factors required for the transcriptional response of all genes inducible by IFN-α and -γ remains to be established. The results presented here, however, emphasize the apparent general requirement of the 91 kDa polypeptide in the primary transcriptional response to both types of IFN.

Key words: cytokines/gene expression/mutant/signal transduction

Introduction

The interferons (IFNs) were initially defined by their ability to interfere with viral replication but are known to affect many cell functions including cellular differentiation, growth and the immune response. IFN-α/β (Type I IFNs) and IFN-γ (Type II IFN) interact with cells through distinct receptors and activate the transcription of overlapping sets of genes (reviewed by Pestka *et al.*, 1987; Staeheli, 1990; Sen and Ransohoff, 1993).

Binding of IFNs-α/β to their receptor(s) causes the rapid activation of a latent cytoplasmic transcription factor, E or ISGF3 (interferon-stimulated gene factor 3) (Dale *et al.*, 1989a; Levy *et al.*, 1989). Active ISGF3 migrates to the nucleus, where it binds to *cis*-acting DNA elements termed ISREs (IFN-stimulable response elements), located within a few hundred base pairs 5′ of the transcriptional initiation sites of interferon-inducible genes. ISGF3 has been shown to be the primary transcriptional activator for IFN-α inducible genes (Kessler *et al.*, 1988a,b; Levy *et al.*, 1988, 1989; Dale *et al.*, 1989a,b; Bandyopadhyay *et al.*, 1990). It is formed from α and γ subunits which associate after activation of the α subunit (Levy *et al.*, 1989). The α subunit(s) (see Discussion) contains three polypeptides, one of 113 kDa (p113) and the 84 and 91 kDa (p84 and p91) products of alternative splicing (Fu *et al.*, 1990, 1992; Kessler *et al.*, 1990; Schindler *et al.*, 1992a). The 48 kDa (p48) γ subunit alone is capable of binding to an ISRE, but its affinity for such motifs increases 25-fold after association with the α subunit (Kessler *et al.*, 1990). Treatment of cells with Type I IFNs leads to the phosphorylation on tyrosine of all three ISGF3α components (Schindler *et al.*, 1992b). A protein tyrosine kinase (Tyk2) is involved in the signal transduction pathway (Velazquez *et al.*, 1992). Transcription through this pathway constitutes a primary response, in that it operates through the activation of pre-existing factors. It has become increasingly clear, however, that all of the components of ISGF3 are themselves inducible in response to IFNs-α or -γ (Schindler *et al.*, 1992b; Veals *et al.*, 1992).

The primary IFN-γ signalling pathway(s) has, until very recently, been less well understood. An immediate early IFN-γ response factor (GAF) has, however, been identified which binds to the GAS (gamma activation sequence) element. This element was first identified in the 5′ flanking region of the GBP gene (Decker *et al.*, 1991a,b; Lew *et al.*, 1991), but has now been implicated in the IFN-γ response of a number of other genes (see Discussion). Recently GAF has been shown to be a 91 kDa DNA binding protein which is recognized by antibodies to the 91 kDa component of the α subunit of ISGF3 (Shuai *et al.*, 1992).

A genetic approach to the dissection of the IFN-α signalling pathway has been developed in the human fibroblast cell line 2fTGH (Pellegrini *et al.*, 1989). In this cell line the *Escherichia coli* gene encoding the drug selectable marker *gpt* (guanine/hypoxanthine phosphoribosyltransferase) is tightly controlled by the promoter of the predominantly IFN-α/β-inducible gene, 6-16. This allows selection for or against IFN-α/β-mediated expression of *gpt*. This, in turn, has permitted the use of this cell line to obtain and complement mutants in the IFN-α/β response pathway. Recessive mutants in five complementation groups, U1–U5, affecting the IFN-α/β response have been isolated to date (Pellegrini *et al.*, 1989; John *et al.*, 1991; McKendry *et al.*, 1991; S.J.Holland, J.John, G.R.Stark and I.M.Kerr, unpublished). Surprisingly, the defects in three of these—U2 to U4—also affect the IFN-γ response (John *et al.*, 1991; McKendry *et al.*, 1991). Mutant U1A (originally coded 11.1) has been complemented with the gene and cDNA coding for the protein tyrosine kinase Tyk2 (Velazquez

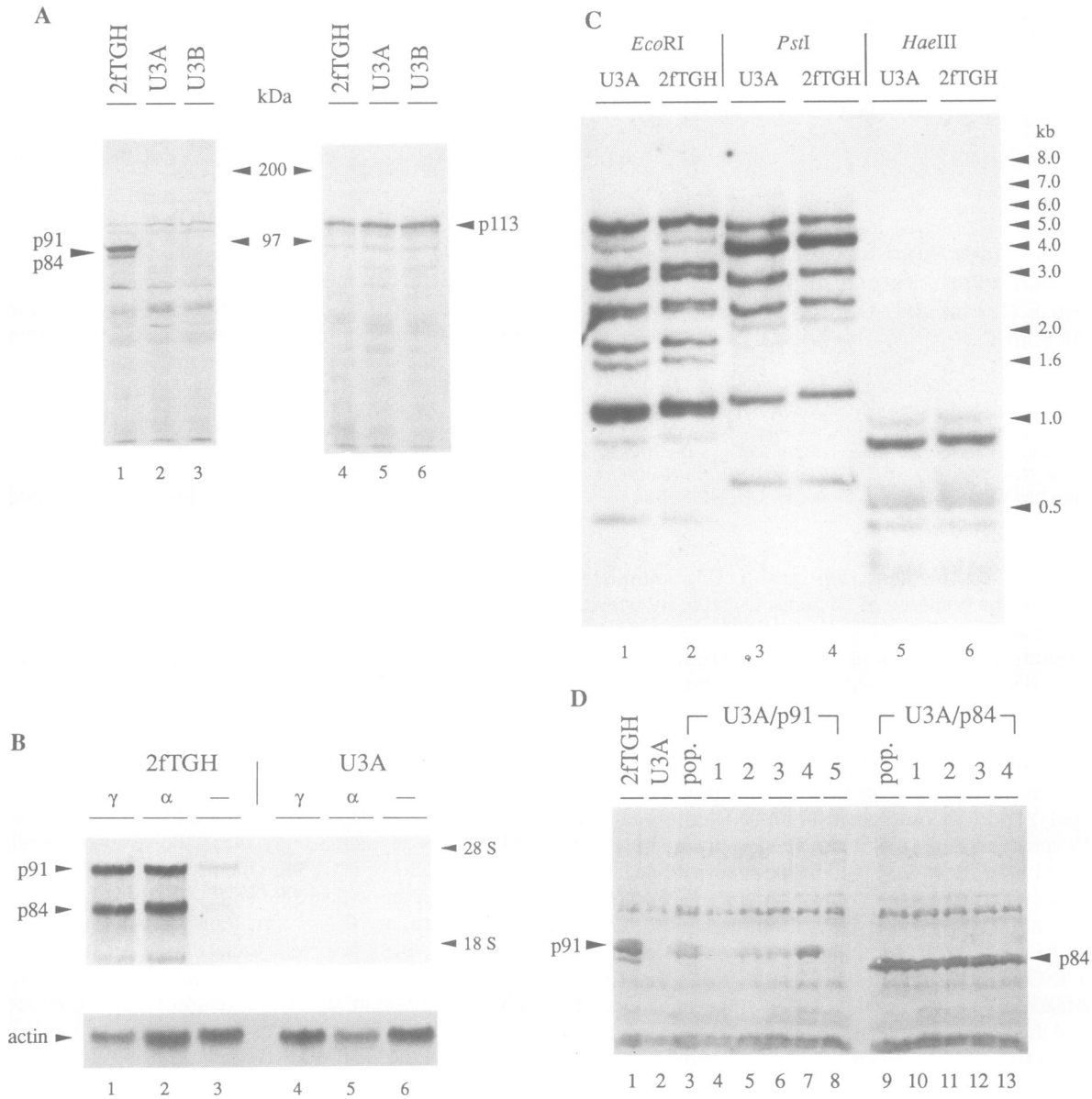

Fig. 1. Characterization of U3 mutants and expression of p84 and p91 in transfectants. (**A**) Western analysis (see Materials and methods) of whole cell extracts from mutants U3A and B and parental 2fTGH cells with antisera to p84/91 (left blot) and p113 (right blot). (**B**) Northern analysis (see Materials and methods) of cytoplasmic RNA from 2fTGH and U3A cells hybridized with a p91 cDNA fragment. A loading control was provided by subsequent hybridization of the Northern blot to actin. Cells were treated with IFN-α for 6 h or IFN-γ for 18 h. The positions of the rRNAs are indicated. (**C**) Southern analysis (see Materials and methods) of genomic DNA from 2fTGH and mutant U3A cells digested with the indicated restriction enzymes and hybridized against a p91 cDNA fragment. The fragment positions of a DNA molecular weight marker (1 kb ladder, BRL) are indicated. (**D**) Western analysis of whole cell extracts from 2fTGH, U3A and U3A transfected with either p91 or p84 expression plasmids using antiserum to p84/91. Transfectants were analysed as a population (pop.) or as single cell clones (numbers). Single cell clones of transfected U3A expressing high levels of the appropriate polypeptide (clone no. 4 for U3A/p91 and clone no. 1 for U3A/p84) were used throughout unless otherwise stated.

et al., 1992) and mutant U2A with a cDNA for the p48 γ subunit of ISGF3 (M.Müller, C.Laxton, J.E.Darnell,Jr, D.E.Levy, G.R.Stark and I.M.Kerr, unpublished). The complementation of mutants in group U3 by the expression of cDNAs encoding the p84 or p91 components of ISGF3 is described here.

Results

Group U3 mutants are defective in the expression of the p91 and p84 components of ISGF3

Mutants in complementation group U3 were previously shown to be defective in the synthesis or activation of the α subunit of ISGF3 (McKendry *et al.*, 1991). Therefore, whole cell extracts were analysed by Western transfer with antisera to p84, p91 and p113 of ISGF3 (Fu *et al.*, 1992; Schindler *et al.*, 1992a,b). Those from mutants U3A and B had normal levels of p113 (Figure 1A, lanes 4–6) but lacked p91 and p84 (Figure 1A, lanes 1–3). Extracts were also assayed for the corresponding alternatively spliced mRNAs encoding p84 and p91 by Northern transfer (Figure 1B). In wild-type 2fTGH cells both mRNAs were expressed at a low constitutive level (Figure 1B, lane 3) and strongly induced by IFN-γ or -α (Figure 1B, lanes 1 and 2). Neither mutant expressed detectable mRNA for either polypeptide without or with IFN-γ or -α treatment

(Figure 1B, lanes 4–6). Southern analysis (Figure 1C) of genomic DNA digested with a variety of restriction enzymes and probed with a cDNA for p91 failed to detect any difference between parental (2fTGH, lanes 2, 4 and 6) and mutant cells (U3A, lanes 1, 3 and 5). Results essentially identical to those in Figure 1C were obtained with two additional mutants in the same complementation group (U3B and C). Accordingly, each of these mutants appears defective in the expression of the products of the p84/91 gene but lacks any obvious rearrangement or deletion in that gene.

Complementation of U3 mutants

Constructs expressing p91 or p84 were stably transfected separately or together into mutant U3A and the phenotype examined. U3A cells transfected with either plasmid showed a growth pattern in selective medium identical to that of parental 2fTGH cells, i.e. IFN-α-dependent cell growth in medium containing aminopterin (HAT) or death in 6-thioguanine (6TG) selection (Pellegrini *et al.*, 1989). This provided confirmation of the nature of the defect in members of complementation group U3 and proof of the functional activity of the p91 and p84 expression constructs.

In parallel, U3A was complemented with a cDNA library in an episomal shuttle vector (Murphy *et al.*, 1992) using back selection for the wild-type phenotype of IFN-α-dependent growth in HAT medium. Isolation of a pool of low molecular weight DNA from the complemented cells yielded a full-length cDNA encoding p91 (M.Müller, C.Laxton, G.R.Stark and I.M.Kerr, unpublished).

Consistent with the restoration of the IFN-α response in cells transfected with either p91 or p84, either component is able to restore both the ability to form ISGF3 and the IFN-α response of a number of inducible genes.

Restoration of the ability to form ISGF3 and the inducibility of the γ subunit (p48) of ISGF3 in U3 mutants

Of the IFN-inducible EMSA (electrophoretic mobility shift assay) complexes reported to date (reviewed by Levy and Darnell, 1990; Kerr and Stark, 1991; Veals *et al.*, 1991), two are detected with extracts from a variety of cell types when a 39 bp oligonucleotide containing the 9-27 ISRE is used as a probe (Parrington *et al.*, 1993): (i) ISGF3 activatable by IFN-α (apparent as a doublet, arrowed lanes 9, 12 and 15, Figure 2) and (ii) the p48 γ subunit of ISGF3, which binds an ISRE probe without induction, but is increased in concentration in response to treatment with either IFN-α or -γ (arrowed lanes 9, 10, 12, 15 and 16, Figure 2). The other bands are not specific since they are not competed by a 50-fold excess of unlabelled probe (Figure 2, lanes 1–4). Neither specific complex was induced in response to either IFN in extracts from mutant U3A cells (Figure 2, lanes 5–7). Comparable amounts of ISGF3 of indistinguishable mobility were, however, found in extracts from wild-type cells and from mutant cells expressing either p91 or p84 (Figure 2, lanes 9, 12 and 15). [The ISGF3 doublet is thought to reflect differences in phosphorylation (Bandyopadhyay and Sen, 1992)]. That ISGF3 containing either p91 or p84 is active is suggested by the general restoration of the IFN-α response in both types of transfected cells (see below).

The IFN-α inducibility of the p48 γ subunit of ISGF3 absent in mutant U3A (Figure 2, lane 6) was restored by either p91 or p84 (lanes 15 or 12), whereas only p91 was

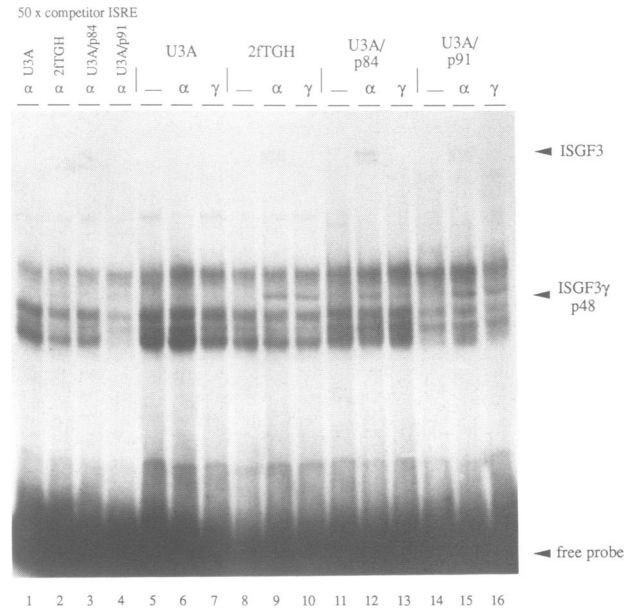

Fig. 2. EMSA of ISGF3 and ISGF3γ (p48) in whole cell extracts from IFN-treated parental 2fTGH cells, U3A mutant and U3A stably expressing p84 or p91. Extracts were prepared from cells with or without treatment for 6 h with 500 I.U./ml of IFN-α or for 18 h with IFN-γ (500 I.U./ml). Incubation was with a 39 bp end-labelled oligodeoxynucleotide probe containing the 9-27 ISRE (see Materials and methods). DNA–protein complexes were separated by electrophoresis (see Materials and methods). The first four lanes show competition with a 50-fold excess of unlabelled 9-27 oligonucleotide for the IFN-α treated extracts.

effective in restoring the IFN-γ response (lanes 16 and 13; and J.Briscoe, M.Müller and I.M.Kerr, unpublished). These data complement similar results obtained in experiments in which the mRNA for the p48 γ subunit rather than p48 *per se* was assayed (Figure 4A, lanes 4, 8, 12, 16). Further evidence that p91 but not p84 restores the IFN-γ response is provided below.

Restoration of the transcriptional response to IFNs in U3 mutants

The initial studies were carried out on populations of transfected cells. These yielded only a partial γ-response even with cells transfected with both p91 and p84 constructs. The partial response reflected a less than optimal expression of p91. Accordingly, the remainder of the work was carried out with individual clones of cells expressing either p91 or p84 to levels comparable to those observed with induced wild-type cells. p84/91 expression was routinely monitored at the mRNA level in parallel with the assay of individual mRNAs (Figures 3–5) and at the protein level by Western transfer (e.g. Figure 1D). Results were confirmed for more than one clone and, where possible, with transfected populations.

The results of RNase protection analyses showing the induction of a wide spectrum of mRNAs in response to IFN-α or -γ in 2fTGH, mutant U3A and mutant U3A cells transfected with either p84 or p91 expression clones, are presented in Figures 3–5 and summarized in Table I. Included are examples of mRNAs inducible only by IFN-α (IFI-56K), only by IFN-γ (Class II HLA DRA) and those inducible to varying degrees by either IFN (Table I). The RNase protection probes for IFN-inducible genes are listed in Table II. Induction of the Class I HLA genes was monitored by fluorescence activated cell scanning (FACS).

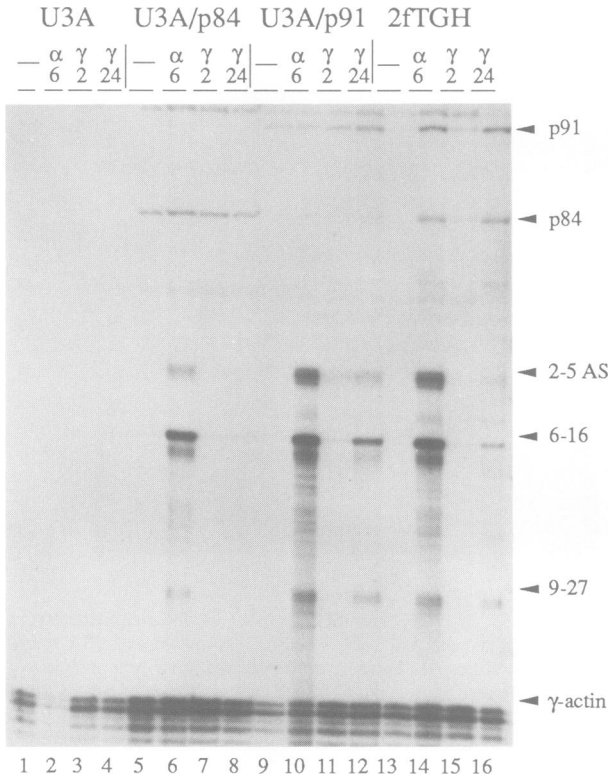

Fig. 3. Restoration of the transcriptional responses to IFNs-α and -γ in mutant U3A cells expressing p91 or p84. Expression of the 2-5A synthetase (2-5 AS), 6-16 and 9-27 mRNAs in mutant U3A cells, U3A transfectants and parental 2fTGH cells in response to IFN-α or -γ. The time (in hours) of treatment with 500 I.U./ml of IFN-α or -γ is indicated above the relevant lanes. Constitutive expression of p84 or p91 mRNAs in transfected U3A cells was comparable to the levels of IFN-induced p91 and p84 mRNAs in 2fTGH cells. Assay of the mRNAs was by RNase protection (Materials and methods) with the protection of γ-actin serving as an internal loading control. Details of the protection probes are given in Table I.

The IFN-α response. The IFN-α response is primary for all of the genes studied (Table I). As expected from the EMSA data and the restoration of IFN-α-dependent growth in selective medium, expression of either p91 or p84 restored the IFN-α-response of genes known to be mediated through interaction of ISGF3 with an ISRE. For example, there was no detectable IFN-α induction of 6-16, 9-27, 2-5A synthetase (Figure 3, lane 2) and IFI-56k mRNA (Figure 5, lane 2) in mutant U3A, but the IFN-α response was restored in U3A expressing p91 (Figures 3 and 5, lane 10) or p84 (Figures 3 and 5, lane 6). In all cases, however, levels of induced mRNAs in cells expressing p84 were consistently slightly lower (compare lanes 6 and 10, Figures 3 and 5). This was true despite comparably high levels of expression of p84 and p91 assayed as protein (as in Figure 1D) or mRNA. [The constitutive levels of expression of the transfected p84 (lanes 5–8) or p91 (lanes 9–12) mRNAs were similar to the IFN-induced levels in 2fTGH lanes 14 and 16, Figures 3 and 5).] An extreme example of the reduced IFN-α response in the presence of p84 compared with p91 was provided by the GBP gene, for which the IFN-α response is thought to be mediated through an overlapping GAS/ISRE promoter element (Decker *et al.*, 1991a; Lew *et al.*, 1991) (see Discussion). Either no detectable response or only a much reduced and delayed response was observed (Figure 4). This result is not peculiar to a single clone of transfected cells (Figure 4A, lanes 6, 10 and 14). It has been confirmed in a population of transfectants expressing p84 to high levels (Figure 4B, lanes 2,3 and 7,8). (Unlike p91, expression of p84 mRNA and polypeptide to high levels is not a problem and is readily obtained in populations of transfectants.) It would appear, therefore, that uniquely amongst the IFN-α-inducible genes analysed to date, the expression of p84 polypeptide is not sufficient to restore a substantial GBP response. The nature of the response element governing ISGF3γ (p48) expression is not known. Once again the IFN-α response is restored by either p84 or p91 (Figure 4A, lanes 6 and 10). The very weak induction of IRF-1 (e.g. Figure 5, lanes 6, 10 and 14) which does not involve an ISRE is dealt with below (Discussion).

The IFN-γ response. The nature of the motifs and factors mediating the primary and secondary responses of many genes to IFN-γ in different cell lines remains to be rigorously established (reviewed: Kerr and Stark, 1991; Williams, 1991). The response of the IRF-1 gene is primary (Figure 5, lanes 11 and 15; see also Pine *et al.*, 1990; Kanno *et al.*, 1993; Sims *et al.*, 1993); that of the Class II HLA DRA gene is secondary (Figure 5, lanes 12 and 16 and reviewed: Kara and Glimcher, 1991) and the kinetics of induction of the 9-27, 6-16 and 2-5A synthetase genes by IFN-γ in the HT1080-derived cell lines used here are consistent with a secondary response (Figure 3, lanes 11,12 and 15,16). The kinetics of induction of the GBP gene in these cells are also more consistent with a secondary response (Figure 4A, lanes 11,12 and 15,16; Figure 4B, lanes 4 and 5), despite the fact that the IFN-γ response of this gene through GAF and the GAS element is primary (Decker *et al.*, 1989; Shuai *et al.*, 1992). In fact, the distinction between a primary and secondary response may be blurred if a common factor(s), itself inducible by IFNs (e.g. p91), is involved. Despite this apparent complexity, the IFN-γ response of all of these genes was restored in cells expressing p91 but not p84 (Table I): a result consistent with a requirement for p91 in a common primary IFN-γ response. Thus, in mutant U3A there was no detectable IFN-γ induction of 6-16, 9-27, 2-5A synthetase, ISGF3γ (p48), GBP, IRF-1 or HLA Class II DRA (lanes 3 and 4, Figures 3, 4A and 5). The response was restored in U3A expressing p91 (lanes 11 and 12, Figures 3, 4A and 5) but not in U3A/p84 (lanes 7 and 8, Figures 3, 4A and 5; lanes 9 and 10, Figure 4B).

Discussion

The mutants in complementation group U3 are defective in the p91 and p84 components of ISGF3α (Figure 1A). A hybridization probe derived from the p91 cDNA showed the apparent complete absence of their mRNAs (Figure 1B). Analysis of Southern transfers failed to detect any major deletion or rearrangement in the corresponding genes in at least three independently isolated members of this complementation group (Figure 1C and unpublished data). A very low level of the p91 mRNA was, however, consistently observed in response to prolonged IFN-α treatment in U3A cells expressing high levels of p84 (e.g. Figure 4A, lane 6). This would be most consistent with a mutation in the promoter rendering it essentially inactive in U3A, but allowing a low level of transcription in the presence

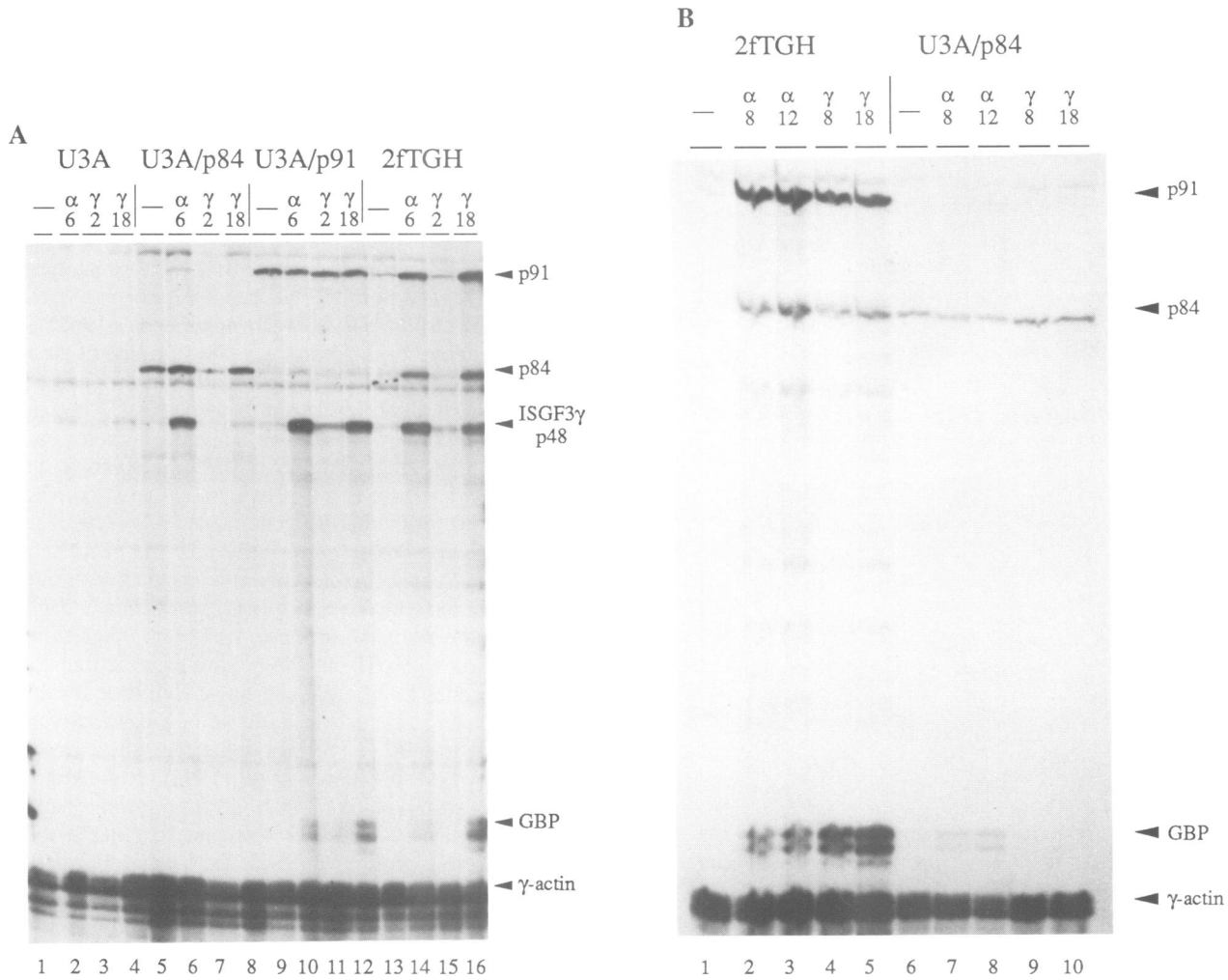

Fig. 4. Analysis of IFN-dependent expression of GBP and ISGF3γ in parental 2fTGH, mutant U3A and U3A cells expressing p84 or p91. (**A**) Treatment was with 500 I.U./ml of IFN-α or -γ for the time (in hours) indicated above the lanes. A very low level of p91 mRNA was detectable in response to IFN-α treatment in U3A cells transfected with p84 (see Discussion). (**B**) Reduced IFN-α response of GBP in U3A transfected with p84. GBP expression was analysed in a population of U3A/p84 transfectants. Treatment was with 500 I.U./ml of IFN-α as indicated (in hours). Assay of the mRNAs including those for p84, p91 and γ-actin as an internal control was by RNase protection as in Figure 3.

of high levels of ISGF3 generated in response to IFN-α in cells transfected with p84. This in turn would imply that the IFN inducibility of the p84/91 gene involves a positive autoregulatory loop. The possibility that the mutation is in a gene required for the production of a factor required for the expression of the p84/91 gene cannot, however, be excluded. Even the detection of point mutations in both alleles of the p84/91 gene would be inconclusive without evidence for consequent lack of function. The conclusions reached here are, however, dependent only on the ability of p91 and p84 to complement and not upon the nature of the defect *per se*.

From the complementation of the mutants, the EMSA and the restoration of the IFN-α response of a number of ISRE containing genes (Figures 3–5) it can be concluded that an active ISGF3α subunit can be formed by the combination of p113 with either p91 or p84. The stoichiometry of the complexes remains uncertain. It is possible, for example, that p113 can combine with homodimers of p91 or p84, but that in wild-type cells the heterodimer is preferred. In this connection, although there appears to be full restoration of the IFN-α response in cells capable of expressing only p91 the levels of IFN-α-induced mRNA in cells expressing only p84 appeared consistently lower (Figures 3–5). This suggests that ISGF3 complexes containing p84 rather than p91 may be slightly less effective in activating transcription from a classical ISRE.

Induction of the GBP gene is regulated through overlapping ISRE and GAS elements. The precise contribution of each to the IFN-α response is not yet clear. Accepting the ability of p84 to form active ISGF3, the basis for the relatively poor IFN-α response of the GBP gene in cells expressing only p84 (Figure 4) remains to be established. There are several possible explanations and it will require substantial further work to determine which is correct.

The promoter/enhancer of the IRF-1 gene lacks an obvious ISRE (Kanno *et al.*, 1993; Sims *et al.*, 1993). Nevertheless the defect in the primary IFN-α response of this gene in U3A (cf. Figure 5) is complemented by p91 (M.Müller, G.R.Stark and I.M.Kerr, unpublished). This suggests a role for p91, alone or with other components, in mediating an IFN-α response through a motif other than an ISRE, analogous to that by AAF through the GBP/GAS motif (Decker *et al.* 1991a; Kanno *et al.*, 1993). Additional evidence for an ISRE-independent IFN-α response has been

provided by the analysis of the induction of IRF-1 by IFN-α in cells lacking a functional p48 ISGF3γ subunit (Haque and Williams, 1992).

p84 is without activity in the IFN-γ response of any gene analysed (Figures 3–5, Table I). It is extremely unlikely that the inactivity of the p84 product reflects a fortuitous mutation(s) exclusively affecting the IFN-γ response in the p84 cDNA construct, as two independently isolated cDNAs—one derived from an active p91 construct—yielded similar results. Details of these constructs and further biochemical and functional characterization of the p91 and p84 products will be reported elsewhere (K.Shuai, G.R.Stark, I.M.Kerr and J.E.Darnell, submitted).

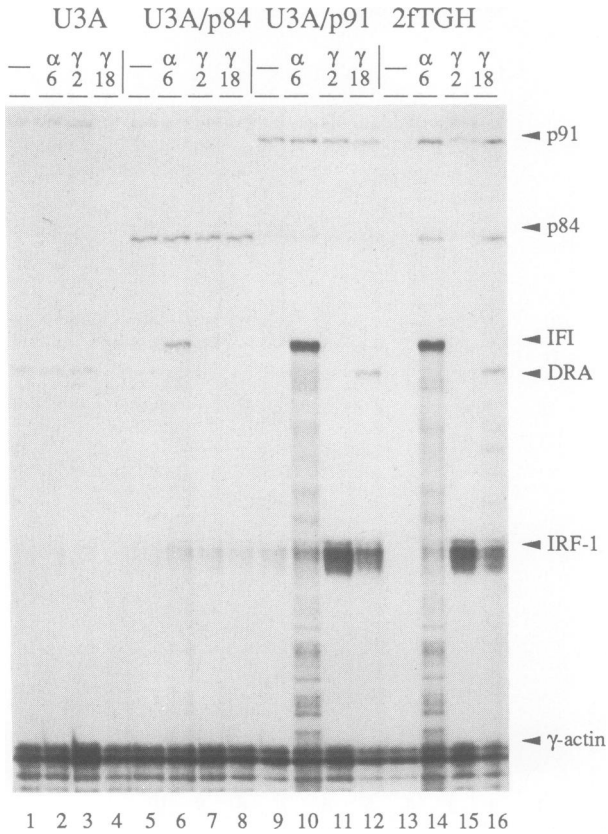

Fig. 5. Response to IFNs-α and -γ of IRF-1, IFI-56k and Class II HLA DRA in complemented U3A cells. Cells (2fTGH, mutant U3A and U3A expressing either p91 or 84) were treated with 500 I.U./ml of IFN-α or -γ as indicated (in hours). RNase protection assay of the mRNAs was as in Figures 3 and 4.

Recent results have implicated p91 in mediating the primary IFN-γ response of the GBP gene through the GAS element. In addition p91 is rapidly phosphorylated on tyrosine in response to IFN-γ (Shuai *et al.*, 1992) and is clearly required for the primary IFN-γ response of the IRF-1 gene (Figure 5). Here complementation of the defect in the γ response of a wide spectrum of genes in U3 mutants by p91 (Figures 3–5; Table I) suggests a role for this polypeptide in a common primary IFN-γ response pathway. Further evidence for this is provided by the work of Kanno *et al.* (1993) for the ICSBP gene, Khan *et al.* (1993) for the Ly6E gene and Pearse *et al.* (1993) for the FCγ1 receptor gene. An additional role(s) in secondary pathways alone or in combination with other factors is not, of course, excluded. Even for the primary response, however, other factors in combination with p91 may be required for the induction of different genes in different cell lines. Evidence for this has recently been presented in the case of the primary IFN-γ response of the FCγ1 receptor gene (Igarashi *et al.*, 1993; Pearse *et al.*, 1993; Perez *et al.*, 1993). It is tempting to speculate that p91, in combination with different 'partners', may also play a role in the response to other cytokines. Indeed, there is preliminary evidence for this. Furthermore, activation and expression p91 has been shown to be under developmental control in cells of the macrophage lineage (Eilers *et al.*, 1993).

In more general terms we have previously shown that mutants in a particular response pathway can be readily generated in mammalian cells. The mutants already analysed have provided invaluable not only in identifying components of the response pathways but also in defining their functions. The availability of mutants in additional complementation

Table I. IFN-inducible mRNAs in parental 2fTGH, mutant U3A and mutant U3A cells expressing the p91 or p84 components of ISGF3

Gene	Response to IFN	IFN-inducibility of mRNAs[a]							
		IFN-α (6 h)				IFN-γ (2 h* or 18 h)			
		2fTGH	U3A	U3A/p91	U3A/p84	2fTGH	U3A	U3A/p91	U3A/p84
IFI-56k	α-specific	+	−	+	+	−	−	−	−
6-16	α>>γ	+	−	+	+	+	−	+	−
2-5A synthetase	α>>γ	+	−	+	+	+	−	+	−
9-27	α > γ	+	−	+	+	+	−	+	−
ISGF3γ(p48)	α = γ	+	−	+	+	+	−	+	−
IRF-1	γ > α	+	−	+[d]		+*	−	+*	−
GBP	γ > α	+	−	+	+[e]	+	−	+	−
HLA Class I[b]	γ > α	+	−	+	+	+	−	+	−
HLA Class II DRA	γ-specific	−	−	−	−	+	−	+	−
INV[c]	γ-specific	−	−	−	−	+	−	+	−

[a]Summary of the data presented in Figures 3–5.
[b]Monitored by FACS analysis of cell-surface expression of HLA Class I antigens.
[c]Data not presented.
[d]M.Müller, G.R.Stark and I.M.Kerr, unpublished.
[e]Partial restoration.

Table II. RNase protection probes for IFN-inducible genes

IFN-inducible gene	Inducibility by IFNs	Length of protected fragment (bp)	cDNA fragment[a]	Gene originally described
IFI-56k	α	245	5′-*Eco*RI (−112 to 133)	Wathelet *et al.*, 1986b
6-16	α>>γ	199	*Sty*I−*Sac*I (−55 to 144)	Kelly *et al.*, 1986; Porter *et al.*, 1988
2-5A synthetase	α>>γ	229	*Hinc*II−*Sac*I (475 to 695)	Wathelet *et al.*, 1986a
9-27	α>γ	163	*Bsp*MII−*Bcl*I (−88 to 75)	Reid *et al.*, 1989
ISGF3γ(p48)	α = γ	270	*Sac*I−*Acc*I (1000 to 1273)	Veals *et al.*, 1992
ISGF3αp91/84	α = γ	400 = p91 322 = p84	*Sma*I−*Eco*RI (1949 to 2353)	Schindler *et al.*, 1992a
IRF-1	γ > α	175	5′-*Mro*I (192 to 367)	Maruyama *et al.*, 1989
GBP	γ > α	138	*Hin*dIII−*Xba*I (aa 244 to 290)[b]	Cheng *et al.*, 1986
HLA Class II DRA	γ	236	*Pst*I−*Sau*3A (592 to 828)	Lee *et al.*, 1982

[a]Nucleotide numbers given in brackets refer to their position in the described cDNA.
[b]The GBP fragment corresponds to the indicated amino acids (aa) as predicted by the cDNA sequence (Decker *et al.*, 1989).

groups with characteristics consistent with defects in the primary IFN-α and/or -γ (above and D.Watling, G.R.Stark and I.M.Kerr, unpublished) response should similarly facilitate the further elucidation of the signal transduction pathways involved.

Materials and methods

Cell culture, DNA transfection and selections

Parental 2fTGH and U3 mutant cells have been described previously (Pellegrini *et al.*, 1989; McKendry *et al.*, 1991). All cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and 5 μM L-glutamine. 2fTGH cells and derived mutant cell lines were maintained in hygromycin (250 μg/ml). DNA transfections using calcium phosphate−DNA precipitates were performed according to standard protocols (Ausubel *et al.*, 1992). G418-resistant ($G418^R$) cells were maintained in medium containing 700 μg/ml G418. Selections in HAT (20 μg hypoxanthine, 0.2 μg aminopterin and 20 μg thymidine per ml) and 6-thioguanine (6TG, 30 μM) medium were carried out as described by Pellegrini *et al.* (1989). IFNs were used at 500 IU/ml for the times stated. IFN-α was a highly purified mixture of human subspecies [Wellferon, 1.5×10^8 IU/mg protein (Allen *et al.*, 1982); provided by Wellcome Research Laboratories, Beckenham, Kent, UK]. Recombinant IFN-γ (4×10^7 IU/mg protein) was supplied by Dr G.Adolf, Ernst Boehringer Institut für Arzneimittelforschung, Vienna, Austria.

Plasmids

Full-length cDNAs encoding p91 and p84 (Schindler *et al.*, 1992a) were cloned into the mammalian expression vector pRc/CMV (Invitrogen) containing the neomycin resistance gene for selection of G418 resistant stable cell lines.

Northern analysis

Northern blotting was performed by electrophoresis of cytoplasmic RNA (20 μg RNA per sample) in a 1% agarose−0.6 M formaldehyde gel, transfer to Hybond-N^+ membranes (Amersham) and hybridization at 65°C according to Church and Gilbert (1984). High stringency washes were performed as described by Ausubel *et al.* (1992). The hybridization probe was a 2.18 kb *Pst*I−*Eco*RV fragment (nt 935−3115) of the p91 cDNA (Schindler *et al.*, 1992a) labelled to a specific activity of $>10^8$ c.p.m./μg by using a multiprime DNA labelling system (Amersham).

Southern analysis

Genomic DNA (30 μg) isolated by standard protocols (Ausubel *et al.*, 1992) was digested with appropriate restriction enzymes, fractionated in a 0.8% agarose gel and transferred to Hybond-N^+ membranes (Amersham). Hybridization was carried out as described for Northern analysis using the same multiprime labelled p91 cDNA fragment.

RNase protection assay

Total cellular RNA was prepared from monolayer cells by NP40 lysis and phenol−chloroform extraction (Porter *et al.*, 1988). RNase protections (Melton *et al.*, 1984) were performed using probes synthesized from SP6/T7 transcription vectors. Probes were labelled with [^{32}P]UTP to a specific activity of $2-5 \times 10^8$ c.p.m. per μg of input DNA. $1-3 \times 10^5$ c.p.m. of each probe and 10 μg of cytoplasmic RNA were used in each assay. The probes protected the mRNA fragments listed in Table II. The 9-27 probe has been described previously (Ackrill *et al.*, 1991). The IRF-1 and 6-16 probes were kindly provided by S.Goodbourn. The GBP probe was a gift from T.Decker. The actin probe was a previously described cDNA fragment of human γ-actin which yields a 130 bp fragment on protection (Enoch *et al.*, 1986). The other probes were constructed in pGEM4 vector (Promega) using appropriate fragments from the cDNAs (Table II).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed by a modification of the protocol of Zimarino and Wu (1987). Briefly, whole-cell extracts were prepared by lysing cell pellets in an equal volume of extraction buffer (0.76 M NaCl, 1.5 mM $MgCl_2$, 10 mM HEPES, pH 7.9, 0.1 mM EGTA, 5% glycerol, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonylfluoride). Lysates were snap-frozen on liquid nitrogen or dry ice, subsequently thawed on ice and centrifuged at 100 000 *g* at 4°C for 30 min. The supernatant solution was dialysed against binding buffer (same as extraction buffer but 0.1 M NaCl) for 2 h at 4°C. Protein concentrations were normalized using the Bio-Rad protein assay. Extracts containing 10 μg protein were pre-incubated with 1.2 μl poly(dI-dC) (5.4 mg/ml, Pharmacia) at room temperature for 10 min and 1.25 μg BSA, 0.125 μg polyd$(N)_5$, 0.125 μg *E.coli* DNA, 0.25 μg yeast tRNA, 2% Ficoll 400 and 0.32 ng labelled probe added in binding buffer to a final volume of 20 μl. The reaction mix was incubated at room temperature for 15 min prior to analysis on non-denaturing 6% polyacrylamide gels. The ^{32}P-end-labelled probe was a 39 bp oligonucleotide with the sequence (double-stranded) 5′-TTTACAAACAGC-AGGAAATAGAAACTTAAGAGAAATACA-3′ containing the 9-27 ISRE (underlined) and flanking region (Reid *et al.*, 1989).

Western blotting

Whole cells were lysed in reducing buffer and proteins were separated by SDS-PAGE (Laemmli, 1970). Analysis with antisera was by Western transfer according to standard protocols (Ausubel *et al.*, 1992). The primary antibodies used are described elsewhere (Fu *et al.*, 1992; Schindler *et al.*, 1992a,b). Detection was with anti-rabbit, peroxidase-conjugated, antibody (Dakopatts Glastrup, Denmark) and diaminobenzidine staining.

Analysis by fluorescence activated cell scanning

Cell surface expression of HLA Class I in response to IFNs was monitored by FACS analysis using a pan-Class-I antibody, W6/32, as described by McKendry *et al.* (1991).

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