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Constitutive Production of Alpha and Beta Interferons in Mutant Human Cell Lines

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Alpha and beta interferons control expression of a selectable marker in the human hypoxanthine phosphoribosyltransferase-negative cell line 2fTGH, in which transcription of *gpt* is regulated by the upstream region of an interferon-responsive human gene. Selection of mutagenized 2fTGH cells in hypoxanthine-aminopterin-thymidine medium yielded mutants in one recessive (C1) and two dominant (C2 and C3) complementation groups. The mutants constitutively expressed low levels of beta interferon (C1), alpha interferon (C2), or both (C3).

The group of interferons (IFNs) that includes the alpha IFN (IFN-α) proteins, a protein family encoded by at least 20 genes, and beta IFN (IFN-β), which is encoded by a single gene, make up a vital part of the cell's primary defense against viral infection (for a review, see reference 8). Infection of cells leads to transcriptional induction of IFN-α/β genes (for a review, see reference 15). The secreted IFNα/β interact with neighboring cells to initiate a complex response that inhibits viral replication.

IFN-α/β bind to specific cell surface receptors, leading to the rapid transcriptional induction of many genes. The IFN-binding component of the IFN-α/β receptor(s) has been cloned elsewhere (50), and a *cis*-acting DNA sequence, the interferon-stimulated response element (ISRE), which is located upstream of most IFN-α-inducible genes, has been identified (6, 29, 38, 42). The primary transcription factor ISGF3 is composed of two subunits, ISGF3α and ISGF3γ. Both are present in the cytoplasm of untreated cells (7, 31). Inactive ISGF3α is rapidly activated upon IFN treatment, allowing its association with ISGF3γ and translocation to the nucleus, where it binds to ISREs to activate transcription. ISGF3α is composed of three polypeptides (84, 91, and 113 kDa), whereas ISGF3γ is a single 48-kD polypeptide (12, 26). cDNAs corresponding to all four polypeptides have now been cloned (13, 44, 51). Recent work has shown that the three ISGF3α components have SH2 domains and are rapidly phosphorylated on tyrosine residues in response to treatment with IFN-α (11, 45), resulting in their activation. At least two protein tyrosine kinases of the JAK family are required for the response to IFN-α: Tyk2 (52) and JAK1 (34).

To complement biochemical analysis of this pathway, we set up a system to generate mutant cell lines. A human hypoxanthine phosphoribosyltransferase-negative cell line was transfected with the gene encoding the selectable marker guanine phosphoribosyltransferase (*gpt*) under the control of the IFN-α/β-responsive 6-16 promoter. A cell clone (2fTGH) was isolated in which expression of *gpt* is tightly controlled by IFN-α/β (37). Mutagenized 2fTGH cells selected in 6-thioguanine (6TG) plus IFN have lost their response to IFN; those selected in hypoxanthine-aminopterin-thymidine (HAT) medium express IFN-inducible genes constitutively. Five complementation groups (U1 to U5) of mutants defective in responding to IFN-α/β have been selected so far (21, 22, 32, 37). Genetic complementation of mutant U1A led to the identification of Tyk2, a tyrosine kinase essential for signaling in response to IFN-α (52). U3 and U4 mutants, which are defective in response to both IFN-α/β and gamma IFN (IFN-γ), are complemented by p91 (35) or JAK1 (34), respectively. U2 mutants lack functional ISGF3γ (30) and have partial defects in response to IFN-α and IFN-γ (22). We now report the successful selection with HAT medium of three mutant cell lines in which IFN-induced genes are expressed constitutively.

To select constitutive mutant cell lines, approximately 3×10^7 2fTGH cells were mutagenized once with ICR-191 as described previously (37) and passaged for 16 days before selection in HAT medium without IFN (37). The cells were maintained in eight independent pools throughout mutagenesis and selection. After 2 weeks of selection, 20 resistant colonies were visible. Resistant colonies must express *gpt* in the absence of added IFN but could carry a mutation either in *cis* or *trans* with the exogenous *gpt* genes. All 20 HAT-resistant colonies were ring cloned, and their expression of the endogenous 6-16 gene was evaluated by Northern (RNA) analysis (37). Five clones expressed high levels of 6-16 mRNA in the absence of added IFN-α (data not shown). They were derived from three separate pools of mutagenized cells, and only one mutant was chosen from each pool to ensure that they represented independent mutations. The frequency of obtaining these mutants (designated C1, C2, and C3) was approximately 10^{-7}, which is considerably higher than the frequency of obtaining unresponsive mutants after one exposure to ICR-191 (10^{-8} to 10^{-9}) (32). The difference may reflect more targets in the genome, different selection protocols, or the expected differences in frequencies of obtaining dominant or recessive mutations. All three mutant cell lines were stable in culture; their spontaneous reversion frequencies, measured as the frequency of resistance to 6TG without added IFN, were $<10^{-7}$, making them suitable for complementation analyses.

To test for dominance, each line (resistant to hygromycin) was fused with a puromycin-resistant population of HT1080 cells (32). Colonies of hybrid cells, obtained with a frequency of about 10^{-3}, were ring cloned from each fusion experiment,

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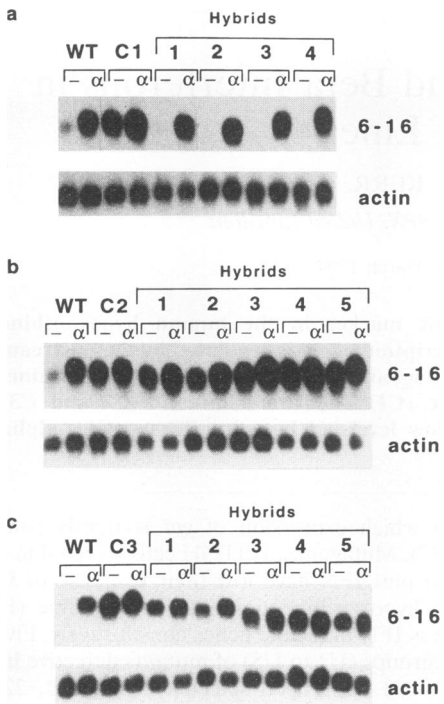

FIG. 1. Dominance analysis of mutants C1, C2, and C3. Equal numbers of mutant (resistant to hygromycin) and HT1080puro^r cells were fused and selected in hygromycin and puromycin. Hybrid colonies were ring cloned, and expression of 6-16 mRNA was examined by Northern analysis. Loading was normalized by subsequently probing with radiolabelled actin cDNA. The 6-16 and actin DNAs used as templates for probe preparation have been described previously (25). −, no treatment; α, IFN-α for 6 h; WT, wild-type 2fTGH cells. (a) C1 × HT1080; (b) C2 × HT1080; (c) C3 × HT1080.

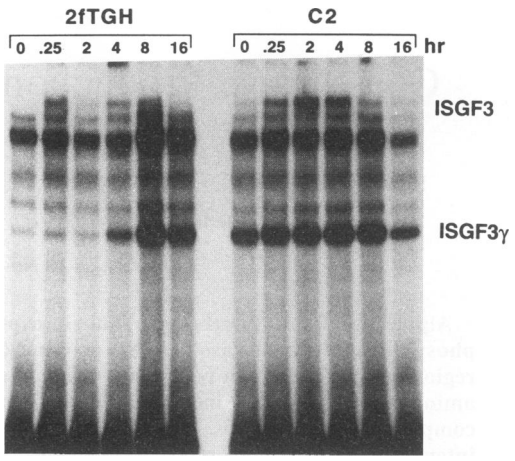

FIG. 2. Gel retardation analyses with whole-cell extracts of 2fTGH and C2 cells after treatment with IFN-α for various times (32). A 39-bp probe (TTTACAAACAGCAGGAAATAGAAACTTAAGAGAAA TACA) representing the ISRE (underlined) and flanking sequences from the IFN-inducible 9-27 gene (36) was used. IFN-inducible complexes containing ISGF3 and ISGF3γ were detected in 2fTGH cells, and both were seen constitutively in C2 cells.

and expression of the 6-16 gene was examined by Northern analysis (37). Examples are shown in Fig. 1. In each of the nine C1 hybrids tested, the basal expression of 6-16 was reduced to wild-type levels, indicating that the mutation in the C1 cell line is recessive (Fig. 1a). In each of the 10 C2 hybrids tested, the high constitutive expression of 6-16 was retained, indicating that the mutation in the C2 cell line is dominant (Fig. 1b). Nine of 10 C3 hybrids also retained high constitutive levels of the 6-16 mRNA, indicating that the mutation in C3 is also dominant (Fig. 1c). The 10th C3 hybrid may have lost the chromosome carrying the dominantly acting gene.

The IFN responses in mutants C1, C2, and C3 were tested by probing Northern transfers with cDNAs representing other IFN-inducible mRNAs. Genes normally expressed in response to IFN-α in 2fTGH cells (6-16, 9-27, and 2′,5′-oligoadenylate synthetase) were expressed constitutively at a high level in the absence of added IFN in all three mutant cell lines (data not shown). This effect appears to be limited to IFN-α/β-inducible genes; expression of HLA-DRA (a gene of the class II major histocompatibility complex) is induced by IFN-γ and not IFN-α in parental 2fTGH cells and in all three mutant cell lines. Expression of all of the IFN-α/β-inducible genes examined can be induced further by IFN-α treatment, indicating that the cells retain the capacity to respond to IFN.

Gel retardation assays were carried out, using as a probe a 39-bp element representing the ISRE and flanking sequences from the IFN-α/β-inducible 9-27 gene (32). This probe detects ISGF3, the primary transcription factor involved in the IFN-α/β-response and, independently, the ISGF3γ subunit (36). After 2fTGH, C1, and C2 cells were treated with IFN-α for various lengths of time, gel retardation assays were carried out with whole cell extracts (Fig. 2) (data not shown for C1). In 2fTGH cells, ISGF3 is detectable within 15 min of IFN treatment and not after 16 h, whereas induction of ISGF3γ is detected after 4 h of IFN treatment, and its level remains elevated at all of the later time points that were examined in this experiment. Untreated C2 cells have a constitutive level of ISGF3 which is increased further after treatment with IFN (Fig. 2). It was not possible to detect constitutive ISGF3 in C1 cells under these conditions. (However, in a different experiment with the same probe and slightly different binding conditions, ISGF3 has been detected in untreated C1 and C3 cells [56]). Both C1 and C2 cells clearly showed constitutive levels of ISGF3γ (Fig. 2) (data not shown for C1 cells). These results are consistent with our present understanding of the function of ISGF3 as the primary transcriptional activator of the IFN-α/β-inducible genes.

The antiviral responses of all three cell lines were compared with that of 2fTGH cells. The assay was carried out with encephalomyocarditis virus as described elsewhere (1), and it was found that virus replication was inhibited in all three mutant cell lines without addition of IFN (data not shown).

The mutants produce IFN constitutively. The mutations in cell lines C1 to C3 could lie in a gene required for the IFN-α/β response or in a gene required for regulating expression of the IFN-α/β genes themselves. To distinguish between these possibilities, an antiviral assay was carried out to determine whether medium conditioned by growing C1, C2, or C3 cells afforded 2fTGH cells any protection against infection with encephalomyocarditis virus. Medium conditioned for four days was assayed in parallel with a dilution series of an IFN-α standard (1). Medium conditioned by C1, C2, or C3 cells gave, respectively, no protection, the same protection as 6 IU of IFN-α per ml, or the same protection as 18 IU of IFN-α per ml. Therefore, C2 and C3 cells both produce IFN.

To determine which types of IFN were produced, all three mutant cell lines were grown in the presence of antibodies to

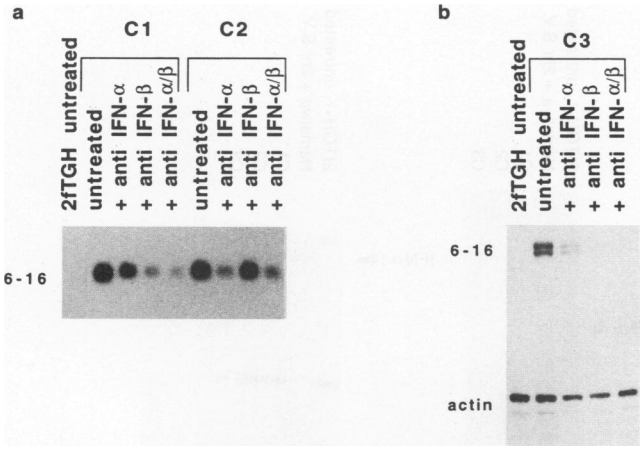

FIG. 3. Growth of constitutive mutants C1, C2, and C3 in antibodies to IFNs. Cells were grown in the presence or absence of antibodies to IFN-α, IFN-β, or a mixture of the two, and the basal level of expression of 6-16 was assayed. Anti-IFN-α sheep polyclonal antibodies raised against a mixture of human IFN-α subspecies were the kind gift of T. Meager (National Institute for Biological Standards and Control, London, United Kingdom). Anti-IFN-β bovine polyclonal antibodies raised against natural human IFN-β were the kind gift of J. Vilček. There was no evidence of cross-reactivity of excess anti-IFN-α with IFN-β or of excess anti-IFN-β with IFN-α. (a) Northern analysis of RNA from C1 and C2 cells after growth in the presence of antibodies. Ethidium bromide was mixed with the RNA sample to allow visualization of the RNA after electrophoresis. This analysis showed that each lane was loaded equally. (b) RNase protection analysis of RNA from C3 cells after growth in the presence of antibodies. Loading was normalized by including an actin probe in the hybridization.

either IFN-α, IFN-β, or a mixture of the two, and, after 5 days, the expression of 6-16 mRNA in the antibody-treated cells was compared with its expression in untreated cells (Fig. 3). Antibodies to IFN-α were effective in reducing the constitutive expression of 6-16 mRNA in C2 cells, whereas antibodies to IFN-β had no effect, indicating that this cell line produces IFN-α (Fig. 3a). With C3 cells, antibodies to IFN-α resulted in a twofold reduction (the autoradiograph was scanned with a densitometer, and values were normalized for loading by comparison with actin), antibodies to IFN-β resulted in a fivefold reduction, and both antibodies together resulted in a sevenfold reduction in the level of 6-16 mRNA (Fig. 3b), indicating that C3 cells produce both IFN-α and IFN-β, but predominantly IFN-β. A more surprising result was obtained with cell line C1. Even though medium conditioned by C1 cells did not protect 2fTGH cells against viral infection, growth of this cell line in antibodies to IFN-β did result in a substantial reduction in the constitutive expression of 6-16 mRNA (Fig. 3a), indicating that C1 cells produce levels of IFN-β that are too low to inhibit viral replication in the assay used but high enough to stimulate their own IFN-α/β response in an autocrine fashion.

Detection of specific IFN mRNAs. The IFN-α proteins are a family of proteins encoded by over 20 functional genes. In a dominant mutant cell line producing IFN-α constitutively, such as C2, the mutation could be in *cis*, in the promoter region of one of the IFN-α genes, or in *trans* with the IFN-α genes. To distinguish between these possibilities, we determined whether one or more IFN-α subtypes are produced. RNA from mutant C2 cells was hybridized with antisense RNA probes representing different IFN-α subtypes, and an RNase protection assay was carried out (2). Antisense RNA probes transcribed from fragments of the coding sequences of IFN-α1, -α2, -α4, and -α14 were used in this assay. IFN-α4 and -α14 mRNAs were detected, but IFN-α1 and -α2 mRNAs were not (Fig. 4). The presence of more than one IFN-α subtype suggests strongly that the mutation in the C2 cell line is in *trans* with the IFN-α genes. None of the four IFN-α mRNAs were detected in C1 or C3 cells.

Antibodies to IFN-β substantially reduce expression of the 6-16 gene in mutants C1 and C3, indicating that these cell lines produce IFN-β. However, an RNase protection assay failed to detect IFN-β mRNA in any of the cell lines in the absence of an IFN-β inducer (data not shown), indicating that very low levels of IFN-β mRNA may be sufficient to generate enough IFN-β for biological activity.

In summary, we have isolated three cell lines which regulate IFN production abnormally. Recessive cell line C1 produces some IFN-β, dominant cell line C2 produces some IFN-α proteins, and dominant cell line C3 produces both types of IFNs (IFN-α and IFN-β), predominantly IFN-β. The regulation of IFN production is a subject of considerable interest. Mutational analyses of the IFN-β promoter have revealed four positive regulatory domains, PRD I to IV, that are involved in transcriptional induction (for reviews, see references 9 and 15), and a number of putative positive regulators which specifically bind to these sequences have been identified (9, 14, 20, 24, 28, 33, 53). Dominant mutant cell line C3 could carry a mutation which causes a single positive regulator of both the IFN-α genes and the IFN-β gene to be constitutively active since, as shown in Fig. 3b, antibody to either IFN-α or IFN-β reduces expression of the 6-16 gene. These results also eliminate a mutation in *cis* in the promoter of only one IFN gene as the cause of the dominant phenotype.

Many lines of experimentation have indicated that IFN-β expression is also under negative regulation (4, 10, 16, 17, 40, 57, 59, 60). *cis*-acting regions have been defined, and protein factors which are candidate negative regulators have been described elsewhere (18, 23). Recessive mutant C1 may have lost a negative regulator of IFN-β expression.

Less work on expression of the IFN-α genes has been carried out. The promoters of these genes show significant homology to that of the IFN-β gene (55), and in many cases IFN-α and IFN-β genes seem to be induced together, although this is not always the case, e.g., primary human fibroblasts produce a mixture of IFN-α and IFN-β after induction by virus, but only of IFN-β after induction by double-stranded RNA (5, 19). The expression of individual IFN-α subtypes shows distinct subtype-specific induction, e.g., infection of mouse L cells with Newcastle disease virus activates transcription of IFN-α4 but not IFN-α6 whereas, in infected macrophages, both of these genes are efficiently induced. We failed to detect IFN-α1 and -α2 in mutant cell line C2 but were able to detect the constitutive expression of both IFN-α4 and -α14. This difference is likely to reflect differences in the regulation of these pairs of subtypes. Comparison of their promoters shows no obvious basis for the difference, but it is likely that subtle changes may be involved, e.g., a two-nucleotide difference is responsible for the differential inducibility of the IFN-α4 and -α6 genes (39). It is not known why there are so many different IFN-α subtypes. Understanding subtype-specific expression may help to answer this question.

The 2fTGH cell line, constructed to enable selection of mutants in the response pathway to IFN-α/β has now been used successfully to select for both unresponsive and constitutive mutants. The ability to select both for and against expres-

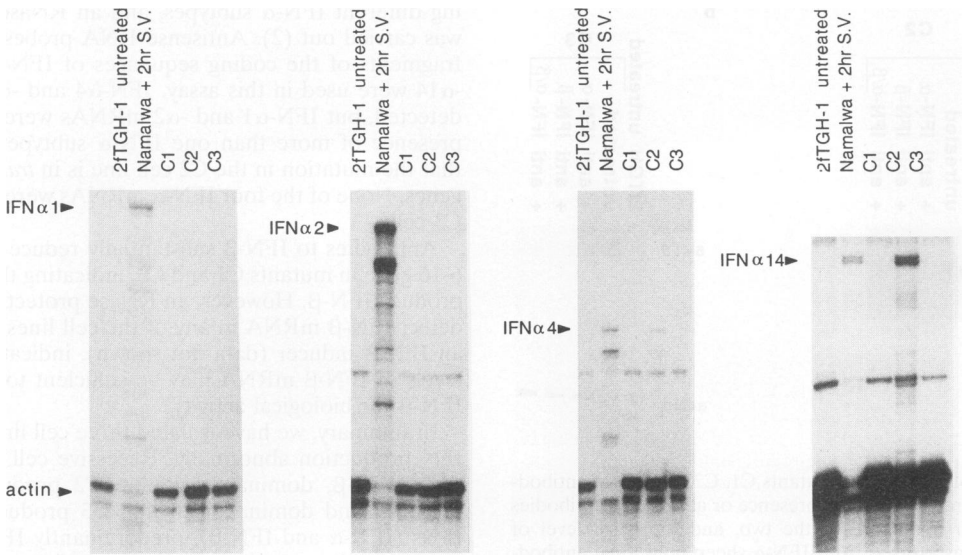

FIG. 4. RNase protection assays to detect mRNAs for different IFN-α subtypes in 2fTGH, C1, C2, and C3 cells. The assays were carried out as described by Ackrill et al. (2). RNA from Namalwa cells treated for 2 h with Sendai virus was used as a positive control (the kind gift of Stephen Goodbourn). Plasmids containing IFN-α1, IFN-α2, IFN-α4, and IFN-α14 fragments were kindly provided by C. Weissmann (49). The IFN-α1 fragment, which was provided in the vector pSP64, was removed by digestion with *Eco*RI and *Hin*dIII and then ligated directly into the vector pGEM4. The probe yielded a protected fragment of 300 bp. The IFN-α2, IFN-α4, and IFN-α14 fragments, which were provided in the vector pSP65, were removed by digestion with *Eco*RI and *Hin*dIII and were then ligated directly into the vector pGEM3. The probes yielded protected fragments of 272, 195, and 260 bp, respectively. Specific probes representing IFN-α1, IFN-α2, IFN-α4, and IFN-α14 were used in separate protection experiments, as indicated. An actin probe was induced in the hybridization for normalization.

sion of *gpt* means that recessive mutants such as C1 can be complemented by transfection and subsequent back selection. Cloning by complementation has been achieved for the unresponsive mutant cell line U1A (52). Further analysis of the constitutive mutant cell lines and cloning of the complementing genes should lead to the isolation of novel factors involved in regulating IFN production or to the further understanding of known factors.

The defects in the constitutive mutants that we have isolated cause abnormal production of IFN. Therefore, the affected genes are not likely to be involved directly in the response to IFNs. To allow selection of constitutive mutants that are affected directly in their IFN response, it should be possible to mutagenize a cell line that does not respond to IFN, such as a mutant in the U3 or U4 complementation group, and then select in HAT medium.

The genetic approach we have taken is proving to be invaluable in furthering understanding of how cells respond to IFNs (34, 35, 54). Selective systems similar to those that we have used should prove useful in analyzing pathways of response to other cytokines and growth factors. Indeed, recent work has suggested there will be a great deal of overlap among such pathways, since kinases and transcription factor subunits similar or identical to those that function in the IFN pathways are also essential for responses to many other extracellular signaling proteins (3, 27, 41, 43, 46–48, 58).

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