MOLECULAR AND CELLULAR BIOLOGY, Nov. 1989, p. 4605–4612
0270-7306/89/114605-08$02.00/0

Vol. 9, No. 11

Use of a Selectable Marker Regulated by Alpha Interferon To Obtain Mutations in the Signaling Pathway†

SANDRA PELLEGRINI,‡ JOSEPH JOHN, MOIRA SHEARER, IAN M. KERR, AND GEORGE R. STARK*
Imperial Cancer Research Fund, Lincoln's Inn Fields, London, WC2A 3PX, United Kingdom

Received 13 June 1989/Accepted 14 August 1989

We have selected mutations in genes encoding components of the signaling pathway for alpha interferon (IFN-α) by using a specially constructed cell line. The upstream region of the IFN-regulated human gene 6-16 was fused to the *Escherichia coli* guanine phosphoribosyltransferase (*gpt*) gene and transfected into hypoxanthine-guanine phosphoribosyltransferase-negative human cells. These cells express *gpt* only in the presence of IFN-α. They grow in medium containing hypoxanthine, aminopterin, and thymidine plus IFN and are killed by 6-thioguanine plus IFN. Two different types of mutants were obtained after treating the cells with mutagens. A recessive mutant, selected in 6-thioguanine plus IFN, was completely resistant to IFN-α but responded normally to IFN-γ and, unexpectedly, partially to IFN-β. A constitutive mutant, selected in hypoxanthine-aminopterin-thymidine alone, was abnormal in expressing endogenous genes in the absence of IFN. Both types revert infrequently, allowing selection for complementation of the defects by transfection.

We are now beginning to understand how DNA elements and protein factors interact positively and negatively to achieve finely tuned regulation of gene expression in eucaryotic cells (33). Although appreciable progress has been made in mammalian systems at the biochemical level, detailed genetic analysis of the factors that regulate transcription has come largely from work with *Saccharomyces cerevisiae*, where mutants can be obtained much more easily. Yeast mutants can be characterized readily by analyzing the progeny of sexual matings, phenotypically defined structural or regulatory mutations can be cured by molecular complementation, and new phenotypes can be obtained by gene replacement (46, 47). The complexity of the genome and the diploid complement of DNA present major obstacles to genetic analyses in mammalian cells. Mutations of a few genes, selected on the basis of resistance to toxic agents, have been used to study the nature of spontaneous or induced mutations in *cis* (6, 9, 49–52). However, no mutations inactivating factors that stimulate mammalian gene expression in *trans* have been obtained, even though they would be invaluable in complementing biochemical analyses of the control networks. Using an approach similar to ours, Hofstetter et al. (25) did obtain mutants in which calcitonin-regulated genes were abnormally expressed in the absence of inducer. The defect in these mutants has not yet been identified.

We now report successful use of a genetic approach to isolate regulatory mutations in the signaling pathway for alpha interferon (IFN-α). Several laboratories have defined regulatory elements of genes activated by IFN-α and -β and have described factors that interact with these elements (10, 12, 30, 41, 43). The human gene 6-16 responds well to IFN-α and -β, and the level of 6-16 mRNA is very low in untreated cells (18, 26). Rapid induction of transcription leads to substantial accumulation of 6-16 mRNA in treated cells, to about 0.1% of the polyadenylated RNA within a few hours. The tightly regulated 6-16 promoter was placed 5′ of a selectable marker and used to construct a cell line whose phenotype could be controlled by IFN-α. The *Escherichia coli gpt* gene (36) is especially advantageous for this purpose because defined selection protocols, originally developed for studying mammalian hypoxanthine-guanine phosphoribosyltransferase (HPRT) genes, allow not only forward selection in hypoxanthine-aminopterin-thymidine (HAT) medium, but also back selection with 6-thioguanine (6TG) for gpt^- cells (17, 37, 51). After transfection of human $HPRT^-$ cells with an IFN-inducible 6-16 *gpt* recombinant plasmid, mutants were selected either with HAT minus IFN or with 6TG plus IFN. We isolated one mutant in which IFN-α is unable to induce expression of any gene tested and a different mutant in which significant levels of mRNAs encoded by IFN-inducible genes accumulate even in the absence of IFN.

MATERIALS AND METHODS

Recombinant plasmids. A previously cloned 8.7-kilobase (kb) DNA fragment containing the entire human 6-16 gene (27, 41) was digested with *Nci*I, and the 1.8-kb fragment was isolated. This fragment contains the 5′ control elements, the TATA box, the cap site, and 40 base pairs of exon I from the 6-16 gene. After addition of *Hin*dIII linkers, the 1.8-kb fragment was ligated to the unique *Hin*dIII site of the promoterless plasmid pSV0gpt (37), and a recombinant containing the insert in the correct orientation (p1.8gpt; Fig. 1) was isolated. pSV2hyg has been described previously (8).

Cells, transfection, and selection. $HPRT^-$ HT 1080 cells, kindly provided by Peter Goodfellow (Imperial Cancer Research Fund), were grown in Dulbecco's modification of Eagle medium with 10% heat-inactivated fetal calf serum. The cells were passaged in 30 μM 6TG before transfection. Hyg^r colonies were generated by cotransfecting 1 μg of pSV2hyg and 10 μg of p1.8gpt per 5×10^5 cells, using calcium phosphate (20). Forty-eight hours later, the cells were plated in medium containing hygromycin (250 μg/ml), and colonies appeared 8 to 10 days later. To analyze the phenotypes of these transfectants, 2×10^5 cells from a pool of Hyg^r colonies were seeded into a 10-cm dish in HAT medium (15 μg of hypoxanthine, 0.2 μg of aminopterin, and 10 μg of thymidine per ml) with or without 500 IU of a highly

* Corresponding author.
† This paper is dedicated to Arthur Kornberg on the occasion of his seventieth birthday, with appreciation for his kindness and dedication to excellence.
‡ Present address: Unité INSERM 276, Institut Pasteur, 75724 Paris Cedex 15, France.

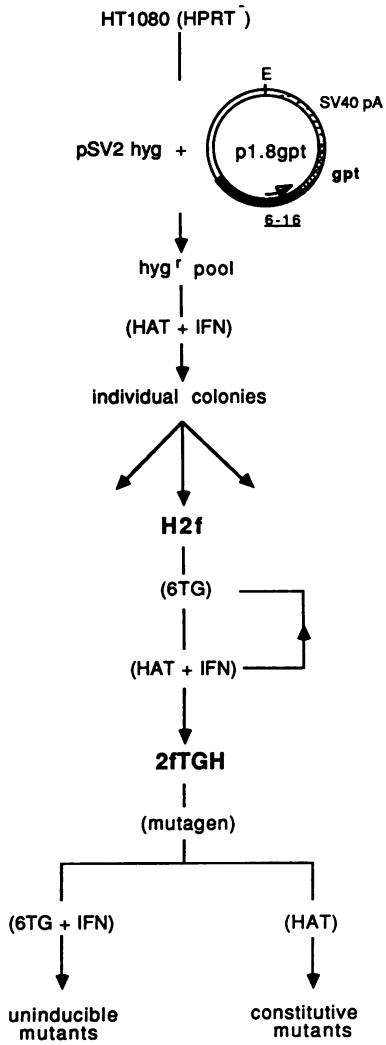

FIG. 1. Schematic representation of the strategy used to isolate the 2fTGH cell line and to select mutants. Construction of the p1.8gpt plasmid and details of transfections and selections are in Materials and Methods. The second sequential selection in HAT + IFN, then 6TG, is indicated by the upward arrow. Constitutive mutants express the *gpt* gene in the absence of IFN, and uninducible mutants fail to express it in response to IFN.

purified mixture of IFN-α per ml (5), 10^8 IU/mg of protein, kindly provided by Wellcome Research Laboratories. Control pSV2gpt transfectants grew independently of IFN, whereas survival of the p1.8gpt transfectants was reduced by about 90% in the absence of IFN. Individual colonies were isolated from cultures seeded at a low density in HAT + IFN and passaged in HT medium (no aminopterin) before culturing in ordinary medium. Their resistance to 6TG was assessed by seeding 2×10^5 cells in the presence or absence of IFN. Ten p1.8gpt sublines were tested. The cloning ability of six was low in 6TG (1 to 5% of control), probably due to a low uninduced level of *gpt* sufficient to metabolize 6TG. Four sublines survived in 6TG but exhibited high frequencies of spontaneous resistance to 6TG + IFN, ranging from 10^{-3} to 10^{-4}. To learn how resistant variants arose, the chromosomal DNA of (6TG + IFN)^r cells derived from one p1.8gpt clone was analyzed with a *gpt* probe. No *gpt* sequences were detected in Southern transfers, and the cells had lost resistance to HAT + IFN as well as to hygromycin. Therefore, these resistant cells probably arose by deletion of the transfected DNA, as observed previously in a number of other studies (6, 50, 51). On the basis of this result we maintained the four 6TG^r cell lines in medium containing hygromycin to select against deletions.

To identify a line with a tightly regulated phenotype, about 10^5 cells from each of the four lines were cultured in HAT + IFN for 4 to 5 days until confluent. After passage first in HT and then in nonselective medium for four population doublings, 10^5 cells per 10-cm dish were exposed to 6TG and survivors were allowed to reach confluence. After a second cycle of selection in HAT + IFN, then 6TG (Fig. 1), we measured the frequencies of spontaneous resistance in HAT alone or in 6TG + IFN. One of the four p1.8gpt lines formed colonies in 6TG + IFN at a high frequency (10^{-3}), and two had only a 10-fold reduction in the number of resistant colonies. The fourth line, 2fTGH, gave a very low frequency of colonies resistant to 6TG + IFN ($<10^{-7}$) and gave no HAT-resistant variants. Reversion frequencies were determined by seeding a total of 10^7 cells at 2×10^5 or 4×10^5 per 10-cm dish. The cells were fed every 5 days, and all dishes were stained 21 to 25 days later. To determine the cloning efficiency, cells were seeded in duplicate at densities of 10^3 and 5×10^3 per 10-cm dish. Colonies were stained and counted after 10 to 15 days. The plating efficiency of HT 1080 cells at these low densities is about 10 to 20% in normal medium. Comparable efficiencies were obtained for 2fTGH cells in normal, HAT + IFN, and 6TG media.

Mutagenesis. Cells were plated at a relatively low density, 4×10^5 per 10-cm dish, and 16 h later fresh medium containing 2 μg of ICR 191 (Polysciences Inc.) per ml was added for 2 h. The cells were rinsed twice with serum-free medium and incubated in medium plus hygromycin. Cell survival after treatment was determined by measuring cloning efficiencies in regular medium, and conditions which caused 70 to 90% killing were chosen. Following recovery for 7 to 9 days, selection was applied to the mutagenized population by adding 6TG and 250 IU of IFN-α per ml. After 4 to 5 days (long enough for wild-type cells to be killed), the cultures were fed with medium containing 6TG without IFN. IFN was added again as soon as colonies appeared. It is not clear whether this pulsed exposure to IFN is necessary for successful selection. Mutant B-HAT was isolated from 2fTGH-1 cells (a Neo^r derivative of 2fTGH) treated with 0.2 μg of *N*-methyl-*N*-nitrosoguanidine (Sigma) per ml for 2 h. After treatment, the cells were allowed to recover, were passaged regularly in normal medium for about 11 days, and then were seeded at 3×10^5 per 10-cm dish in medium plus HAT.

Antiviral assay. Cells were seeded in 96-well microtiter plates at 5×10^4 per well, and the following day the cultures were incubated for 16 to 24 h with twofold serial dilutions of IFN-α. Encephalomyocarditis virus and Semliki Forest virus were used at multiplicities of infection from 100 to 0.1 PFU/cell, and the cytopathic effect was assessed 3 to 4 days later by microscopic examination before and after staining of the cells.

Cell fusions. HPRT$^-$ HT 1080 cells resistant to G418 (Neo^r) were fused with 11,1 cells in suspension by using polyethylene glycol (molecular weight, 1,000), and the cells were seeded in 24-well plates. Selection was applied 1 day later by adding hygromycin and G418 or HAT + IFN to the medium. Colonies in each well were pooled for further analysis.

RNA and DNA analyses. Total RNA was extracted from

subconfluent cultures with guanidinium thiocyanate (32). Ten micrograms of RNA per track was fractionated in 1.2% agarose gels containing 2.2 M formaldehyde, transferred to nitrocellulose, and hybridized under standard conditions (32). A human β-actin cDNA probe was used to estimate the amount of RNA in each track. Ten micrograms of genomic DNAs or the indicated amounts of plasmid DNAs were digested with restriction endonucleases, run in a 0.8% agarose gel, and, after depurination, transferred to nitrocellulose and hybridized under standard conditions (32). Fragments of plasmid DNAs to be used as probes were gel purified and electroeluted twice and were labeled to a specific activity of more than 10^8 cpm/μg by random priming (16). Probes were used at concentrations of 2×10^6 to 3×10^6 cpm/ml. A 480-base-pair *Bgl*II-*Eco*RV fragment from pSV2gpt was used as the *gpt* probe; the ISG-54 probe (29) was an *Eco*RI fragment about 600 base pairs long from exon 3, kindly provided by David Levy (Rockefeller University, New York, N.Y.). The other probes have been described previously (26). Recombinant human IFN-β was a gift of Triton Biosciences Inc., and recombinant human IFN-γ (2×10^7 IU/mg) was a gift of G. R. Adolph, Ernst-Boehringer-Institut für Arzneimittel-Forschung, Vienna, Austria.

Binding assay. Recombinant human IFN-α_2 was labeled with ^{125}I (specific activity, 35 to 184 μCi/μg, 2×10^8 IU/mg [48]). Cells were seeded in six-well plates at 8×10^5 per well and, 20 to 24 h later, were rinsed twice at 20°C with binding buffer (Dulbecco-modified Eagle medium, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] buffer [pH 7.8], 0.1% bovine serum albumin, 0.1 μM KI). Binding buffer (0.6 ml per well) containing labeled IFN-α_2 was added, and the plates were rocked gently for 90 min at 20°C. The cells were cooled rapidly, rinsed six times with ice-cold binding buffer, and dissolved in 2 ml of 0.1 M NaOH–2% Na_2CO_3 for gamma counting. Binding specificity was determined in parallel incubations containing a 100-fold excess of unlabeled IFN at each concentration of labeled IFN.

RESULTS

Construction of a selectable cell line. To construct a plasmid with IFN-inducible *gpt* (p1.8gpt; Fig. 1), the simian virus 40 promoter of pSV2gpt was exchanged for a 1.8-kb upstream fragment of 6-16 DNA known to mediate transcriptional induction by IFN-α (Materials and Methods). The fragment contains the initiation site for mRNA synthesis and 40 nucleotides of the untranslated first exon (41). As recipient cells, we used an HPRT$^-$ derivative of the human fibrosarcoma line HT 1080 (11, 42) with a spontaneous frequency of reversion in HAT of less than 10^{-7} (our data, not shown). HT 1080 cells are not sensitive to the antiproliferative effect of IFN-α and express 6-16 mRNA well after IFN treatment (see below). They were cotransfected with the dominant selectable marker pSV2hyg plus either p1.8gpt or pSV2gpt as a control. Pools of 200 Hyg^r colonies from each transfection were expanded and subsequently challenged with HAT, either alone or in the presence of 500 IU of IFN-α per ml (Fig. 1). Individual (HAT + IFN)^r colonies from each pool were then grown in nonselective medium.

To identify a colony whose HAT^r phenotype was absolutely dependent on IFN (*gpt* expression tightly regulated), we challenged each with 6TG. This purine analog, commonly used to select HPRT$^-$ mutants, is converted to a toxic nucleotide by bacterial *gpt*. Cells expressing *gpt* constitutively should not survive in 6TG, while cells that depend on IFN for *gpt* expression should grow (Fig. 1). Initial results

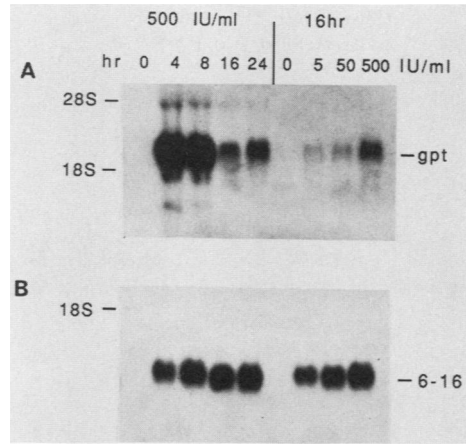

FIG. 2. Induction of *gpt* and 6-16 mRNAs by IFN-α in 2fTGH cells. Northern transfers of 10 μg of total RNA from subconfluent cells, untreated (0) or treated with IFN-α (500 IU/ml) for the indicated times or for 16 h with the indicated amounts of IFN, were hybridized to a *gpt* probe (A). After washing, the same filter was hybridized again to a 6-16 probe (B). The signals in each track were similar with a β-actin probe (data not shown). The positions of rRNAs are indicated.

indicated that the 6TG-resistant lines obtained had high frequencies of spontaneous resistance to 6TG + IFN (10^{-3} to 10^{-4}), probably due to deletion of the transfected *gpt* genes (see Materials and Methods). Therefore, hygromycin was added to all culture media routinely to increase the stability of the transfected DNA. It was difficult to identify a clone of cells with a phenotype tightly regulated by IFN. To do so, we subjected four independent Hyg^r 6TG^r transfectants to two rounds of a double selection regime (Fig. 1). One line, 2fTGH, gave no colonies resistant to 6TG + IFN and no colonies able to grow in HAT, from more than 10^7 cells in each case.

Characterization of 2fTGH cells and scheme for selecting mutants. *gpt* mRNA, undetectable in untreated 2fTGH cells, was induced upon IFN treatment in a dose-dependent manner (Fig. 2). The same Northern (RNA) transfer, rehybridized with a 6-16 probe, showed that the level of 6-16 mRNA reached a maximum 24 h after treatment with IFN-α. The difference in relative abundance of the two mRNAs at various times after IFN treatment probably reflects their different stabilities. 6-16 mRNA is very stable, with a half-life greater than 24 h (D. Gewert and I. M. Kerr, unpublished observations). The organization and copy number of p1.8gpt DNA in 2fTGH cells was studied by hybridizing a Southern transfer with a *gpt* probe (Fig. 3). The cellular DNA contains a large insert, as shown by digestion with *Xba*I, which does not cleave within the DNA of p1.8gpt. Digestion with *Eco*RI, which cuts once, reveals a 6.7-kb band that comigrates with the linearized p1.8gpt marker. Similarly, analysis with three other enzymes reveals *gpt*-containing bands of the sizes expected (Fig. 3). We conclude that 5 to 10 copies of full-length p1.8gpt DNA are arranged in tandem in the DNA of 2fTGH. The extra bands detected in the digests with *Eco*RI, *Hin*dIII, and *Bgl*II probably represent a rearranged or deleted sequence.

Uninducible mutants. Mutations in the signaling pathway can easily be distinguished from mutations which affect *gpt* in *cis* since only *trans*-acting mutations will affect levels of the mRNAs for both *gpt* and 6-16. 2fTGH cells were mutagenized with ICR 191, an intercalating agent known to

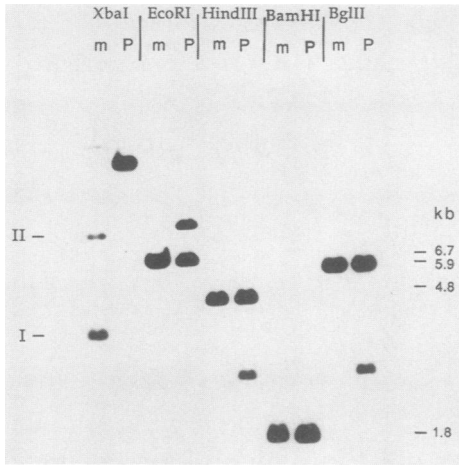

FIG. 3. Analysis of *gpt* DNA sequences in 2fTGH cells. Lanes P, 10-μg samples of genomic DNA from 2fTGH cells were digested with restriction endonucleases, fractionated in a 0.8% agarose gel, denatured, transferred to nitrocellulose, and hybridized with a *gpt* probe. Lanes m, p1.8gpt marker DNA was digested as indicated. The amount of marker DNA (70 pg) is equivalent to about seven copies per genome. *Xba*I does not cut within the plasmid DNA, and therefore forms I and II are visible. *Eco*RI cuts once to linearize the 6.7-kb marker. *Hin*dIII, *Bam*HI, and *Bgl*II cut the marker DNA twice, generating *gpt*-containing fragments of 4.8, 1.8, and 5.9 kb, respectively.

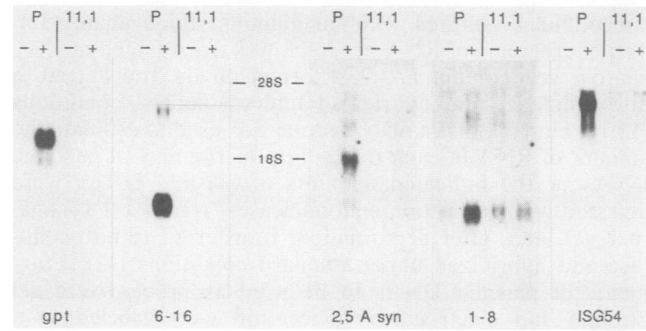

FIG. 4. Induction of mRNAs by IFN-α in 2fTGH (P) and mutant 11,1 cells. Northern transfers of 10 μg of total RNA from untreated cells (−) or from cells treated with 500 IU of IFN-α per ml for 6 h (+) were hybridized with the probes shown. A single transfer was hybridized sequentially with the three probes on the left, and a separate transfer was hybridized with the two probes on the right. The larger RNA detected with the 6-16 probe probably represents a nuclear precursor of 6-16 mRNA.

induce frameshift mutations in bacteria and yeasts and thought to act similarly in mammalian cells (14). The cells were grown for a few days before mutagenesis in 6TG + IFN to eliminate any preexisting mutants. A total of about 10^8 cells were used. In different experiments, the frequency of mutants varied from less than 10^{-7} to about 10^{-6}. Most ring-cloned colonies, expanded in nonselective medium and tested again for resistance to 6TG + IFN, were only partially resistant. The cells grew very poorly because *gpt* was expressed at a low level.

Northern transfers of mRNAs isolated from IFN-treated or untreated cultures were analyzed with *gpt* and 6-16 probes. Some mutants had an induced level of *gpt* mRNA comparable to that of wild-type cells, with enzymatic activity 0 to 10% of the wild-type value (data not shown). In most mutants the level of induced *gpt* mRNA was reduced significantly, whereas 6-16 mRNA expressed from the endogenous gene was regulated normally. Mutants of these types probably arose from structural changes in *cis* that inactivated a few integrated *gpt* genes, thus reducing the level of *gpt* mRNA or the amount of functional enzyme below a critical threshold level. However, one mutant, called 11,1, did not induce either 6-16 or *gpt* mRNA in response to IFN-α at 500 IU/ml (Fig. 4) or 50,000 IU/ml (data not shown). Overexposure of the autogradiograph revealed, in both treated and untreated samples, faint bands at the same intensities as the uninduced messages detected in 2fTGH cells (data not shown). Therefore, the mutation in 11,1 cells affects concomitantly the inducibility, but not the basal expression, of the endogenous 6-16 and transfected *gpt* genes, both of which have identical IFN-responsive promoters.

Properties of uninducible mutant 11,1. Parental 2fTGH cells were protected from infection by encephalomyocarditis virus or Semliki Forest virus by low concentrations of IFN-α. For example, 50% protection from the cytopathic effect of encephalomyocarditis virus was achieved with 4 IU of IFN-α per ml at a multiplicity of infection of 1.5. In contrast, mutant 11,1 was not protected against either virus at a multiplicity of 0.1, even by 10,000 IU of IFN-α per ml. The mutation in 11,1 causes a major block in the pathway for activating expression of several IFN-responsive genes. Induction of the genes for 1-8, 2′,5′-oligo(A) synthetase, and ISG 54 (Fig. 4) and HLA class I (data not shown) was analyzed in Northern transfers of mRNAs from wild-type and mutant cells, untreated or treated for 6 h with 500 IU of IFN-α per ml. The steady-state levels of these mRNAs increased 3- to 20-fold in wild-type cells, but not at all in mutant 11,1. The low level of 1-8 mRNA present in human cells in the absence of IFN (26) was also observed in untreated 11,1 cells, suggesting that the signals responsible for basal and induced 1-8 expression are different.

The induction of 1-8 mRNA by IFN-γ (26) was unimpaired in mutant 11,1 (Fig. 5A). A similar result was obtained for 2′,5′-oligo(A) synthetase mRNA (data not shown), indicating that the IFN-γ signaling pathway is unaffected. Since IFN-α and -β are thought to share a common receptor (34, 40) and since they induce expression of the same genes, it was surprising that 11,1 cells were able to respond to IFN-β by inducing 6-16 mRNA (Fig. 5B), although the level was appreciably lower than in wild-type cells. Upon rehybridization, this Northern transfer also showed that ISG 54 and *gpt* mRNAs were induced by IFN-β in 11,1 cells (data not shown). The levels of these two mRNAs, clearly visible 6 h after treatment with IFN-β, decreased at later times, in contrast with 6-16 mRNA, which is more stable (Fig. 5B).

Binding of IFN-α. Specific binding of iodinated IFN-α_2 (48) to 2fTGH and 11,1 cells was determined as a function of IFN concentration. The Scatchard (44) plot for 2fTGH cells was upwardly concave (inset to Fig. 6), suggesting the presence of high- and low-affinity binding sites. 11,1 cells bound consistently lower levels of radiolabeled IFN-α_2 and appeared to lack the high-affinity component (Fig. 6). Similar observations have been reported by others when the binding of IFN to cells of different sensitivities was compared (2, 3, 23, 54). The data suggest that 11,1 cells are impaired in their ability to bind IFN in a functional way. The mutation could be in a component of the receptor for IFN-α which has not yet been well characterized.

Complementation analysis. The approach used here provides advantages over previous strategies used to isolate mutants in the IFN response pathway (1, 15, 21) since it

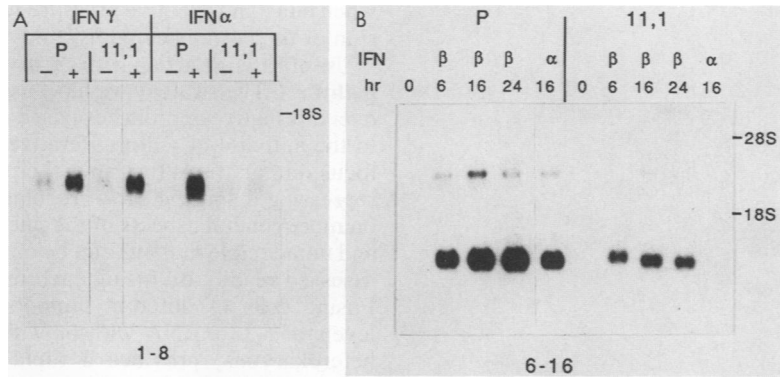

FIG. 5. (A) Induction of 1-8 mRNA by IFN-γ in 2fTGH (P) and mutant 11,1 cells. Subconfluent cultures were treated with 500 IU/ml of either IFN-γ for 16 h or IFN-α for 6 h. Total RNA (10 μg) from untreated (−) or treated (+) cells was analyzed by hybridization with a 1-8 probe. (B) Induction of 6-16 mRNA by IFN-β in 2fTGH (P) and mutant 11,1 cells. Subconfluent cultures were treated with 500 IU of either IFN-α or recombinant IFN-β per ml for the times indicated. Total RNAs (10 μg) from untreated (0) or treated cells were analyzed with a 6-16 probe.

facilitates both genetic analysis and cloning of the affected genes. The dominant or recessive nature of the mutation can be defined by fusing cells and selecting hybrids in appropriate media. If a stable mutant resistant to 6TG + IFN is complemented by the wild-type gene, the inducible *gpt* phenotype will be regained and the cells will survive in HAT + IFN. To date, we have not observed a single spontaneous revertant from about 10^8 11,1 cells cultured in nonselective medium for 2 months. Therefore, we were able to carry out complementation analyses. Neo^r HPRT$^-$ HT 1080 cells were fused with Hyg^r 11,1 cells and selected in HAT + IFN or in hygromycin + G418. The two selections yielded similar numbers of resistant hybrids. Clones from individual wells (two to four per well) were pooled, and restoration of the inducible phenotype was confirmed by analyzing the behavior of four such pools in HAT with or without IFN and in 6TG with or without IFN. All the hybrid pools had the phenotype expected for cells with an IFN-inducible *gpt* gene. Analysis of Northern transfers of mRNAs isolated from the Neo^r Hyg^r hybrids confirmed that *gpt* mRNA was present in IFN-treated cells (Fig. 7), although at a level lower than that of unfused parental cells. These results show that the 11,1 mutation is recessive.

The mutation in 11,1 can also be complemented by chromosomes or DNA from HeLa cells. Lugo and Baker (31) previously observed a frequency of HAT resistance of 10^{-6} when HeLa chromosomes were transfected into HPRT$^-$ HT 1080 cells. Using their procedure and HeLa metaphase chromosomes (five cell equivalents per cell), we observed a very similar frequency of transfer into HPRT$^-$ 11,1 cells: 25 colonies from 3.6×10^7 cells. Our selection was with HAT + IFN, allowing growth of both HPRT$^+$ transfectants and HPRT$^-$ transfectants in which the 11,1 mutation was complemented. Subsequent analysis of eight clones showed that five grew in HAT without IFN (HPRT$^+$) and three did not (HPRT$^-$). Induction of 6-16 mRNA by IFN was restored only in the latter three clones (data not shown), showing that the 11,1 mutation was indeed complemented. The frequency of successful DNA transfer, using 40 μg of DNA per 10^6 cells and the procedure of Goodfellow et al. (19), was much lower, about 10^{-8}. However, the single transfectant ob-

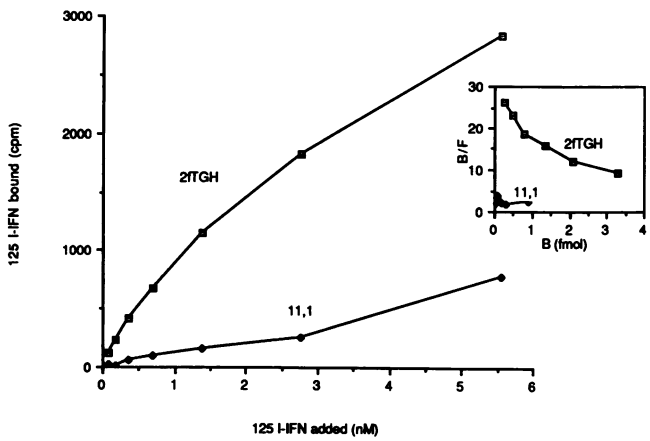

FIG. 6. Binding of ^{125}I-labeled IFN-α_2 to 2fTGH and 11,1 cells. Experiments were performed with 2×10^6 cells for 90 min at 20°C with the indicated concentrations of IFN. Nonspecific binding, determined in parallel assays containing a 100-fold excess of unlabeled IFN, represented about 30% of the total amount bound for 2fTGH and about 75% for 11,1. Specific binding (in counts per minute per 2×10^6 cells) is the difference between the total and nonspecific values. The insert represents the Scatchard plot of the specific saturation curves. One femtomole of labeled IFN = 865 cpm. Bound/free (B/F) is in counts per minute $\times 10^3$.

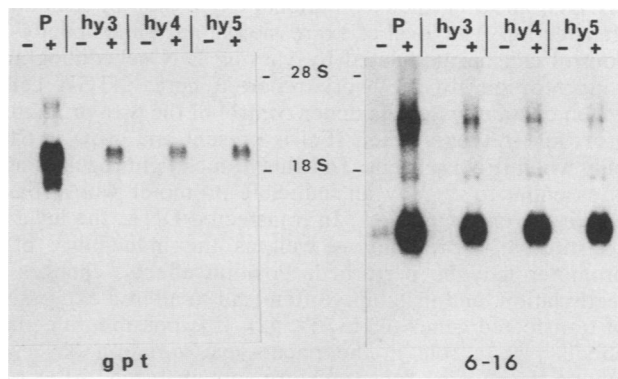

FIG. 7. Analysis of *gpt* and 6-16 mRNAs induced by IFN-α in wild-type × 11,1 hybrid cells. 2fTGH cells (P) and three different pools (hy 3 to 5, each containing three to four Hyg^r Neo^r hybrids from fusion of Neo^r HPRT$^-$ HT 1080 cells with Hyg^r 11,1 cells) were untreated (−) or treated with IFN-α (+), 500 IU/ml, for 6 h. Total RNAs (10 μg) were analyzed with a *gpt* probe. After washing, the same Northern transfer was rehybridized with a 6-16 probe.

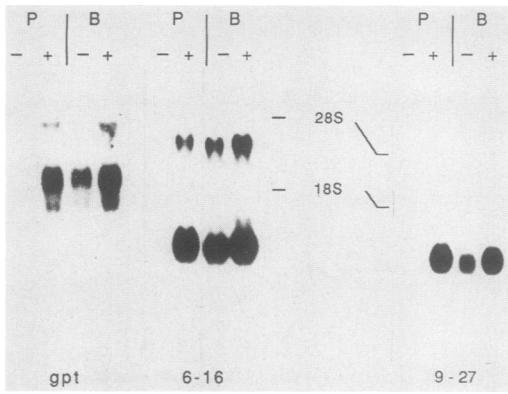

FIG. 8. Analysis of mRNA levels in mutant B-HAT cells. 2fTGH-1 (P) and B-HAT (B) cells were untreated (−) or treated with IFN-α (+), 500 IU/ml, for 6 h. Total RNAs (10 μg) were analyzed by hybridization to the probes shown.

tained was HPRT$^-$ and the 11,1 mutation was complemented, by both of the assays noted above.

A constitutive mutant. To obtain mutants that expressed both *gpt* and 6-16 mRNAs in the absence of IFN, cells were mutagenized with *N*-methyl-*N*-nitrosoguanidine, allowed to recover for 10 to 12 days, and selected in HAT at a maximum density of 3×10^5 per 10-cm dish. Cell density was important as slow-growing colonies which could not be subcloned were often present in dense cultures. For reasons not important to the outcome of the experiment, the cells used were 2fTGH-1, a Neo^r clone of 2fTGH with very similar properties. A successful experiment involving 4×10^7 mutagenized cells yielded two resistant clones, A-HAT and B-HAT, both of which were analyzed for expression of 6-16 and other IFN-responsive genes. A-HAT was not a mutant of the type sought since expression of 6-16 was not altered. Higher levels of 6-16, *gpt*, and 9-27 mRNAs were observed in untreated B-HAT cells compared with untreated 2fTGH-1 cells, and these high basal levels were increased further upon IFN treatment (Fig. 8).

DISCUSSION

Use of a promoter tightly regulated by IFN. The 6-16 promoter can be fused to a variety of different genes or cDNAs, allowing their conditional expression in cells after transfection. The level of expression of any gene under 6-16 control can be modulated by varying IFN scheduling and concentration. In the work reported here, 2fTGH cells, which contain *gpt* genes under control of the 6-16 promoter, grow in HAT only when IFN is present and grow in 6TG only when IFN is absent. To achieve such tight regulation, it is essential to employ an inducible promoter whose basal activity is extremely low. In transfected DNA, the level of constitutive expression as well as the inducibility of a promoter may be perturbed. Position effects, changes in methylation, and instability often lead to altered expression of transfected genes (6, 13, 39, 52). It is possible that such effects play a role in the phenotypic variation observed among the four p1.8gpt transformants analyzed. After two rounds of selection in HAT + IFN and then in 6TG without IFN, to eliminate cells in which the *gpt* gene was not tightly regulated, only one of these four lines (2fTGH) was stable with respect to regulated *gpt* expression and had reversion frequencies of less than 10^{-7} in HAT alone or in 6TG + IFN. The frequency of cells with deletion of *gpt* was low only when constant selective pressure was maintained for expression of the cotransfected hygromycin B gene.

Use of lethal selection with *gpt* to obtain mutants in signaling pathways. The strategy we have used has several advantages over alternative approaches based on selecting cells resistant to the antiviral or antiproliferative effects of IFN, since we focus on mutations that alter the pathways for activating or repressing a specific set of regulatory elements rather than on more general aspects of the phenotype. Both constitutive and uninducible mutants can be obtained. Dominance can be assessed readily by fusing each mutant with parental cells. Fusing cells of different mutants, followed by analyzing levels of 6-16 mRNA with or without IFN in the resulting heterokaryons, provides a rapid way to define different complementation groups. It should be straightforward to clone the wild-type genes by taking advantage of the ability to select for or against expression of *gpt*. Although the low frequency of recessive mutations makes the task laborious, it should also be possible to isolate additional mutants by further screening of mutagenized cells.

A related approach has been used to study calcitonin induction of the urokinase gene, using a cell line made hormone dependent for expression of *gpt* (25). This work, although promising, has not been carried far enough yet to allow its utility to be assessed. Since HPRT$^+$ cells were used, selection for constitutive expression employed mycophenolic acid and xanthine. No selection was attempted for unresponsive mutants, probably because the parental cells had leaky expression of *gpt* in the absence of calcitonin, which would make such selection very difficult. Furthermore, the constitutive mutants obtained were unstable. This property, and the likelihood that revertants would be as leaky as the parental cells, would make it difficult to complement the mutations by transfection, and no such complementation was demonstrated.

Mutants unresponsive to IFN-α. Since HT 1080 cells are nearly diploid (42), the frequency of recessive mutations is expected to be very low, as it may require independent steps to inactivate each allele. One inactivating event might occur spontaneously in a small fraction of the population and the other as a result of mutagenesis (45). Methylation is a candidate for inactivating one allele, but the stability of the mutation and the absence of revertants in 11,1 cells treated with 5′-aza-2′-deoxycytidine (data not shown) argues against inactivation by methylation. The frequency with which we have isolated regulatory mutants is less than 10^{-8}, a reasonable number if both alleles must be inactivated. There are major uncertainties in estimating the frequency to be expected since we do not know how many target genes there are in the signal transduction pathway for IFN-α or how many of these are indispensable for survival. The frequency of inactivating one copy of a gene by mutagenesis is 10^{-4} to 10^{-5} (45, 49), and the squares of these numbers are close to the frequency we have observed.

The approach we have taken may not allow us to isolate mutations in any gene whose function can be provided alternatively by a different gene, and it is easy to imagine that components of other signaling pathways could function in the IFN response to some extent. By varying the concentration of 6TG and the time cells are exposed to it, we may be able to isolate mutants in which the response to IFN-α is reduced but not eliminated completely. It may also be possible to use cell sorting rather than selection (for example, see reference 38), thus avoiding a requirement for complete off-regulation of the indicator gene in the absence of inducer.

The defect in mutant 11,1. Since the 2fTGH cells were mutagenized with ICR 191, a frameshift mutagen (7, 14), the product of the affected gene is likely to be completely absent or severely truncated. Loss of an early function, such as the receptor, would be consistent with the total unresponsiveness of 11,1 cells to IFN-α.

There is a correlation between IFN binding to a high-affinity class of receptors and biological response (2, 4, 24). For a number of cell lines, resistance to IFN correlates with loss of high-affinity binding sites and retention of low-affinity sites (2, 23, 54). In contrast, in other cases, IFN-resistant mutants have been found to retain their high-affinity sites (2, 28, 35, 53). Binding studies suggest that 11,1 cells do not have high-affinity receptors for IFN-α. The most straightforward explanation is that the 11,1 mutation affects the IFN-α receptor, directly or indirectly. The gene for this receptor has not yet been cloned and its subunit structure has not yet been defined. Since the 11,1 mutation can be complemented by DNA, it should be straightforward to clone the affected gene. The combination of a defective mutant and a cloned functional gene should be powerful in elucidating function.

Although previous binding studies indicated that IFN-α and -β share a common receptor (34, 40), the differential antiviral effects of various subtypes of IFN-α (55) indicated that their interactions with cells could be distinguished functionally. Also, differential binding of IFN-α subtypes has been seen in competition assays (22). Our unexpected finding that IFN-β induces expression of IFN-responsive genes in mutant 11,1, albeit relatively poorly, suggests either that IFN-α and -β interact differently with a single type of receptor to permit a partial response to IFN-β, or that there is an additional receptor specific for IFN-β which functions relatively inefficiently.

Constitutive mutants. In the B-HAT mutant, expression of both 6-16 and 9-27 is constitutive. Loss of one allele encoding a repressor, resulting in decreased expression, might be responsible if the concentration of repressor is crucial. Alternatively, the B-HAT mutation might affect an activator, causing it to induce expression of these genes in the absence of IFN. In more recent work (R. McKendry, unpublished data) it has proved possible to isolate two classes of stable constitutive mutants. Culture medium conditioned by one class does not stimulate expression of 6-16 mRNA in 2fTGH-1 cells. In contrast, mutants in the other class produce an extracellular factor that induces 6-16 mRNA. Both classes are protected from virus infection in the absence of exogenously added IFN. Further characterization of these constitutive mutants should reveal the functions involved. By continuing and extending the approaches described here, we hope to define additional major elements of the signaling pathway for IFN-α.

ACKNOWLEDGMENTS

We thank Roslyn McKendry for help with the experiment shown in Fig. 8 and for allowing us to cite her recent data; Trevor Dale, Mark Meuth, Mary Perry, and Jeff Rosen for suggestions and critical comments; and Clare Middlemiss for typing the manuscript.

S.P. was supported by a long-term European Molecular Biology Organization fellowship.

LITERATURE CITED

1. **Affabris, E., C. Jemma, and G. B. Rossi.** 1982. Isolation of interferon-resistant variants of Friend erythroleukaemia cells: effects of interferon and ouabain. Virology **120**:441–452.
2. **Aguet, M.** 1980. High-affinity binding of ^{125}I-labelled mouse interferon to a specific cell surface receptor. Nature (London) **284**:459–461.
3. **Aguet, M., I. Gresser, A. G. Hovanessian, M.-T. Bandu, B. Blanchard, and D. Blangy.** 1981. Specific high-affinity binding of ^{125}I-labeled mouse interferon to interferon resistant embryonal carcinoma cells *in vitro*. Virology **114**:585–588.
4. **Aguet, M., and K. E. Mogensen.** 1983. Interferon receptors. Interferon **5**:1–22.
5. **Allen, G., K. H. Fantes, D. C. Burke, and J. Morser.** 1982. Analysis and purification of human lymphoblastoid (Namalwa) interferon using a monoclonal antibody. J. Gen. Virol. **63**: 207–212.
6. **Ashman, C. R., and R. L. Davidson.** 1987. Sequence analysis of spontaneous mutations in a shuttle vector gene integrated into mammalian chromosomal DNA. Proc. Natl. Acad. Sci. USA **84**:3354–3358.
7. **Berger, H., W. J. Brammar, and C. Yanofsky.** 1968. Spontaneous and ICR191-A-induced frameshift mutations in the A gene of *Escherichia coli* tryptophan synthetase. J. Bacteriol. **96**: 1672–1679.
8. **Blochlinger, K., and H. Diggelmann.** 1984. Hygromycin B phosphotransferase as a selectable marker for DNA transfer experiments with higher eucaryotic cells. Mol. Cell. Biol. **4**:2929–2931.
9. **Brown, R., and J. Thacker.** 1984. The nature of mutants induced by ionising radiation in cultured hamster cells. Mutat. Res. **129**:269–281.
10. **Cohen, B., D. Peretz, D. Vaiman, P. Benech, and J. Chebath.** 1988. Enhancer-like interferon responsive sequences of the human and murine (2′-5′) oligoadenylate synthetase gene promoters. EMBO J. **7**:1411–1419.
11. **Croce, C. M.** 1976. Loss of mouse chromosomes in somatic cell hybrids between HT-1080 human fibrosarcoma cells and mouse peritoneal macrophages. Proc. Natl. Acad. Sci. USA **73**:3248–3252.
12. **Dale, T. C., J. M. Rosen, M. J. Guille, A. R. Lewin, A. C. G. Porter, I. M. Kerr, and G. R. Stark.** 1989. Overlapping sites for constitutive and induced DNA binding factors involved in interferon-stimulated transcription. EMBO J. **8**:831–839.
13. **Davies, R. L., S. Fuhrer-Krusi, and R. S. Kucherlapati.** 1982. Modulation of transfected gene expression mediated by changes in chromatin structure. Cell **31**:521–529.
14. **De Luca, J. G., D. A. Kaden, J. Krolewski, T. R. Skopek, and W. G. Thilly.** 1977. Comparative mutagenicity of ICR-191 to *S. typhimurium* and diploid human lymphoblasts. Mutat. Res. **46**:11–18.
15. **Dron, M., and M. G. Tovey.** 1983. Isolation of Daudi cells with reduced sensitivity to interferon. I. Characterization. Virology **64**:2641–2647.
16. **Feinberg, A. P., and B. Vogelstein.** 1982. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
17. **Fenwick, R. G.** 1985. The HGPRT system, p. 333–373. *In* M. Gottesman (ed.), Molecular cell genetics. John Wiley & Sons, Inc., New York.
18. **Friedman, R. L., S. P. Manly, M. McMahon, I. M. Kerr, and G. R. Stark.** 1984. Transcriptional and posttranscriptional regulation of interferon-induced gene expression in human cells. Cell **38**:745–755.
19. **Goodfellow, P. N., C. A. Pritchard, and G. S. Banting.** 1988. Techniques for mammalian genome transfer, p. 1–8. *In* K. E. Davies (ed.), Genome analysis. A practical approach. IRL Press, Oxford.
20. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
21. **Gresser, I., M. T. Bandu, and D. Brouty-Boye.** 1974. Interferon and cell division. IX. Interferon resistant L1210 cells: characteristics and origin. J. Natl. Cancer Inst. **52**:553–559.
22. **Hannigan, G. E., D. R. Gewert, E. N. Fish, S. E. Read, and B. R. G. Williams.** 1983. Differential binding of human interferon-α subtypes to receptors on lymphoblastoid cells. Biochem. Biophys. Res. Commun. **110**:537–544.
23. **Hannigan, G. E., D. R. Gewert, and B. R. G. Williams.** 1984.

Characterization and regulation of α-interferon receptor expression in interferon-sensitive and -resistant human lymphoblastoid cells. J. Biol. Chem. **259**:9456–9460.

24. **Hannigan, G., and B. R. G. Williams.** 1986. Transcriptional regulation of interferon-responsive genes is closely linked to interferon receptor occupancy. EMBO J. **5**:1607–1613.
25. **Hofstetter, P., Z. Kikinis, M. S. Altus, D. Pearson, and Y. Nagamine.** 1987. A new genetic approach for studying hormonal regulation of urokinase-type plasminogen activator gene expression in LLC-PK_1 cells. Mol. Cell. Biol. **7**:4535–4541.
26. **Kelly, J. M., C. S. Gilbert, G. R. Stark, and I. M. Kerr.** 1985. Differential regulation of interferon-induced mRNAs and c-myc mRNA by α- and γ-interferons. Eur. J. Biochem. **153**:367–371.
27. **Kelly, J. M., A. C. G. Porter, Y. Chernajovsky, C. S. Gilbert, G. R. Stark, and I. M. Kerr.** 1986. Characterization of a human gene inducible by α- and β-interferons and its expression in mouse cells. EMBO J. **5**:1601–1606.
28. **Kessler, D., R. Pine, L. M. Pfeffer, D. E. Levy, and J. E. Darnell, Jr.** 1988. Cells resistant to interferon are defective in activation of a promoter-binding factor. EMBO J. **7**:3779–3783.
29. **Levy, D., A. Larner, A. Chaudhuri, L. E. Babiss, and J. E. Darnell, Jr.** 1986. Interferon-stimulated transcription: isolation of an inducible gene and identification of its regulatory region. Proc. Natl. Acad. Sci. USA **83**:8929–8933.
30. **Levy, D. E., D. S. Kessler, R. Pine, N. Reich, and J. E. Darnell, Jr.** 1988. Interferon-induced nuclear factors that bind a shared promoter element correlate with positive and negative transcriptional control. Genes Dev. **2**:383–393.
31. **Lugo, T. G., and R. M. Baker.** 1983. Chromosome-mediated gene transfer of HPRT and APRT in an intraspecific human cell system. Somatic Cell Genet. **9**:175–188.
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Maniatis, T., S. Goodbourn, and J. A. Fischer.** 1987. Regulation of inducible and tissue-specific gene expression. Science **236**: 1237–1245.
34. **Merlin, G., E. Falcoff, and M. Aguet.** 1985. ^{125}I-labelled human interferons alpha, beta and gamma: comparative receptor-binding data. J. Gen. Virol. **66**:1149–1152.
35. **Mogensen, K. E., M.-T. Bandu, F. Vignaux, M. Aguet, and I. Gresser.** 1981. Binding of ^{125}I-labelled human α interferon to human lymphoid cells. Int. J. Cancer **28**:575–582.
36. **Mulligan, R. C., and P. Berg.** 1980. Expression of a bacterial gene in mammalian cells. Science **209**:1422–1427.
37. **Mulligan, R. C., and P. Berg.** 1981. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. Proc. Natl. Acad. Sci. USA **78**:2072–2076.
38. **Nolan, G. P., S. Fiering, J.-F. Nicholas, and L. A. Herzenberg.** 1988. Fluorescence-activated cell analysis and sorting of viable mammalian cells based on β-D-galactosidase activity after transduction of *Escherichia coli lacZ*. Proc. Natl. Acad. Sci. USA **85**:2603–2607.
39. **Ostrander, M., S. Vogel, and S. Silverstein.** 1982. Phenotypic switching in cells transformed with the herpes simplex virus thymidine kinase gene. Mol. Cell. Biol. **2**:708–714.
40. **Pestka, S., J. A. Langer, K. C. Zoon, and C. E. Samuel.** 1987. Interferons and their actions. Annu. Rev. Biochem. **56**:727–777.
41. **Porter, A. C. G., Y. Chernajovsky, T. C. Dale, C. S. Gilbert, G. R. Stark, and I. M. Kerr.** 1988. Interferon response element of the human gene 6-16. EMBO J. **7**:85–92.
42. **Rasheed, S., W. A. Nelson-Rees, E. M. Toth, P. Arnstein, and M. B. Gardner.** 1974. Characterisation of a newly derived human sarcoma cell line (HT-1080). Cancer **33**:1027–1033.
43. **Rutherford, M. N., G. E. Hannigan, and B. R. G. Williams.** 1988. Interferon-induced binding of nuclear factors to promoter elements of the 2-5A synthetase gene. EMBO J. **7**:751–759.
44. **Scatchard, G.** 1949. The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. **51**:660–672.
45. **Simon, A. E., M. W. Taylor, W. E. C. Bradley, and L. H. Thompson.** 1982. Model involving gene inactivation in the generation of autosomal recessive mutants in mammalian cells in culture. Mol. Cell. Biol. **2**:1126–1133.
46. **Struhl, K.** 1983. The new yeast genetics. Nature (London) **305**:391–397.
47. **Struhl, K.** 1987. Promoters, activator proteins, and the mechanism of transcriptional initiation in yeast. Cell **49**:295–297.
48. **Taylor-Papadimitriou, J., M. Shearer, and D. Griffin.** 1987. Epitopes of human interferon-α defined by the reaction of monoclonal antibodies with α interferons and interferon analogues. J. Immunol. **139**:3375–3381.
49. **Thacker, J.** 1985. The molecular nature of mutations in cultured mammalian cells: a review. Mutat. Res. **150**:431–442.
50. **Thacker, J., P. G. Debenham, A. Stretch, and M. B. T. Webb.** 1983. The use of a cloned bacterial gene to study mutation in mammalian cells. Mutat. Res. **111**:9–23.
51. **Tindall, K. R., L. F. Stankowski, Jr., R. Machanoff, and A. W. Hsie.** 1984. Detection of deletion mutations in pSV2gpt-transformed cells. Mol. Cell. Biol. **4**:1411–1415.
52. **Tindall, K. R., L. F. Stankowski, Jr., R. Machanoff, and A. W. Hsie.** 1986. Analysis of mutation in pSV2gpt-transformed CHO cells. Mutat. Res. **160**:121–131.
53. **Tovey, M. G., M. Dron, K. E. Mogensen, B. Lebleu, N. Mechti, and J. Begon-Lours-Guymarho.** 1983. Characterization of Daudi cells with reduced sensitivity to interferon. II. On the mechanisms of resistance. Virology **64**:2649–2653.
54. **Yonehara, S., M. Yonehara-Takahashi, and A. Ishii.** 1983. Binding of human interferon α to cells of different sensitivities: studies with internally radiolabeled interferon retaining full biological activity. J. Virol. **45**:1168–1171.
55. **Weck, P. K., S. Apperson, L. May, and N. Stebbing.** 1981. Comparison of the antiviral activities of various cloned human interferon-α subtypes in mammalian cell cultures. J. Gen. Virol. **57**:233–237.