Interferon-α-(IFN) Producing CHO Cell Lines Are Resistant to the Antiproliferative Activity of IFN: A Correlation With Gene Expression

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CHO cell lines that constitutively produce the murine interferon-α (IFN-α) subspecies α4 and α6 were constructed. The producer cell lines were protected against viral (vesicular stomatitis virus) infection by the IFN species secreted, but were resistant to the growth inhibitory activity of the IFN species. As compared with α4, the α6 protein displayed a high antiproliferative activity when added to normal CHO cells, which correlates completely with the high antiviral activity of α6 on these cells. Three messenger ribonucleic acid (mRNA) species, which are normally induced in CHO cells by IFN treatment (1-8, 2-5A synthetase, and ISG 15) were constitutively present in CHO producer cell lines. The level of another mRNA (ISG 54), however, was very low in the producer cells as compared with its expression in short-term IFN-treated cells. These data indicate that 1-8, 2-5A synthetase and ISG 15 are not involved in the antigrowth activity of IFN in this system, but rather suggest a function of ISG 54 in this respect.

Interferon-α (IFN-α) comprises a group of highly homologous proteins encoded by a multigene family of at least 15 members [1]. To exert its various biological activities (antiviral, antiproliferative, and immunomodulatory), IFN binds to a specific cell-surface receptor, whereupon the expression of a series of different genes is induced [2]. The function of the protein products of most of these genes is unknown. For some of the proteins, e.g., 2-5A synthetase (2-5A) [3] or metallothionein-IIA [4], which have a known activity, it is difficult to correlate this activity to one of the IFN-specific biological functions. In contrast, the protein product of the IFN-induced Mx gene has been proved specifically to inhibit influenza virus replication in mice [5]. However, in this case, the properties of the Mx protein are not understood.

Several murine (Mu) IFN-α genes were isolated and their protein products characterized [6–8]. The specific antiviral activities of five different subspecies, as measured on mouse cells, were found to diverge considerably. Even larger differences were found when antiviral titers were measured on hamster cells. To obtain sufficient amounts of the various proteins for further characterization, CHO cell lines were constructed that constitutively produce Mu IFN-α proteins. In addition to their function as IFN producers, these cell lines were used to investigate the effects of long-term IFN exposure on cellular functions. In this study, some properties of CHO IFN producer cell lines are described, and the biological response of cells to continuous and short-term IFN exposure correlated with the expression of IFN-induced genes.

METHODS
Cell Culture and Transfection

Cells were propagated in a 1:1 mixture of Ham's F10 and Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum (FCS), penicillin, and streptomycin. Transfections were performed according to the calcium phosphate precipitation technique [9]. Selection of transfectants was done in the presence of G-418 sulphate (Gibco).

Cell Growth Assay

Normal and IFN-producing CHO-9 and CHO-12 cells were grown to confluency and left for two days on medium containing 0.5% FCS, whereupon the cells were seeded at a density of 10^3 cells/cm² in 12 well clusters in medium containing 5% FCS. One day after seeding, IFN was added to the culture medium. The supernatant of IFN-producing CHO cells was used as a source of the various IFN species. The medium was changed every other day (without or with added IFN). At indicated time points, the cell density was determined by the dye uptake method of Kohase et al. [10].

Antiviral Assay

IFN antiviral titers were determined in a cytopathic effect reduction assay using vesicular stomatitis virus (VSV) as a challenge by essentially the method as described by Armstrong [11]. IFN titers on mouse (L929) cells were calculated relative to the NIH reference standard G002-904-511. Titers on hamster (CHO) cells were calculated relative to the activity of Mu IFN-α1 on these cells, which was set at 100% of its activity on mouse cells [6].

Northern Hybridization Analysis

Total cellular RNA was isolated by the guanidinium thiocyanate method [12]; 20 μg RNA was denatured by glyoxal treatment, separated by electrophoresis on a 1% agarose gel, and transferred to a nylon membrane (GeneScreen) using the method described by the manufacturer (NEN, Boston). Filters were hybridized with complementary (c) DNA probes labelled as described [13]. Overnight hybridization at 42° C in 50% formamide and washing of the filters were as described by NEN. Filters were exposed to a Kodax X-Omat AR film at −70° C using intensifying screens.

RESULTS

Establishment of CHO IFN-α Producer Cell Lines

Mu IFN-α subspecies display different specific antiviral activities on mouse cells. The protein showing the highest activity is α4 (1×10^8 IU/mg protein) [8], and the one with the lowest activity α10 (1.5×10^6 IU/mg) [7]. When specific activities of IFN-α's are measured on hamster cells, α6 shows the highest activity (4×10^7 HU/mg) [6, 8, and unpublished results], whereas α4 is 200-fold lower in activity (2×10^5 HU/mg) [8].

CHO cells are frequently used for the introduction of foreign genes to generate cell lines synthesizing large amounts of the protein product of the introduced gene. These cells were used for construction of cell lines with integrated Mu IFN-α genes. To this end, two different CHO cell lines, CHO-9 [14] and CHO-12 [15], were transfected with a coprecipitate of the selection plasmid pKOneo and the IFN expression plasmids pSVα4 or pSVα6 [6]. In these expression plasmids, an IFN-α coding region is placed downstream of the SV40 early promoter and followed by rabbit β-globin 3'-noncoding sequences. After 2 weeks of G-418 selection, single colonies could be isolated. During this period, the α6 producer clones grew at a slower rate than did the α4 clones. This growth inhibition turned out to be transient, because when the clones could be expanded in culture, this difference in growth rate was much less pronounced.

IFN production by the isolated clones was variable. Approximately 5% of the CHO-9 and 15% of the CHO-12 clones secreted $\geqslant 10^3$ IU/ml IFN-α per day. Regardless of the IFN-α subtype, CHO-12 clones produced about four times more IFN than CHO-9 clones (as measured in IU/ml/day). This is due at least partly to the higher cell density of CHO-12 cells, as compared with CHO-9, in confluent cultures. However, CHO-9 cells have the advantage that they can be maintained in a confluent state for a longer time than CHO-12 cells, enabling a longer period of serial overnight IFN collections. The maximal amount of IFN synthesized by selected high-producer CHO-9 and CHO-12 clones, expressed in antiviral units per 10^6 cells or per ml, is shown in Table I; α4 producers secrete four times more IFN (in antiviral units as measured on mouse cells) than α6 producers. These data, combined with the specific antiviral activity of both subspecies on mouse cells (1×10^8 IU/mg for α4 [8] and 2×10^7 IU/mg for α6 [6, 8, and unpublished results]), indicate that comparable amounts of IFN-α4 and IFN-α6 protein are secreted by CHO cell lines. However, as measured in antiviral units on hamster cells, α6 producers synthesize 250-fold more IFN than α4 producers.

TABLE I. Constitutive IFN Production by CHO Cell Lines Transfected With the Mu IFN-α4 or −α6 Gene*

Cell type	Clone	IFN	IFN production*		
			IU/10^6 cells/day	IU/ml/day	HU/ml/day
CHO-9	61	α4	1.6×10^4	1.6×10^4	32
	106	α6	4×10^3	4×10^3	8×10^3
CHO-12	15	α4	3.2×10^4	6.4×10^4	128
	28	α6	8×10^3	1.6×10^4	3.2×10^4

*IFN production as measured on mouse cells in international units (IU) or as arbitrary antiviral units on hamster cells (HU).

Proliferation and Virus Resistance of Established CHO IFN Producers and Short-Term IFN-Treated CHO Cells

The transient cell growth delay of newly generated IFN-producing CHO clones (see previous section) urged us to investigate in more detail the growth characteristics of CHO cells in the presence of IFN. The growth rate of the various CHO-IFN producers was determined and compared with that of control CHO cells and CHO cells incubated with exogenous IFN-α4 or -α6. The resulting growth curves are depicted in Figure 1 and clearly show that control CHO cells are sensitive to the antiproliferative activity of IFN, whereas the established IFN-producing CHO cells grow as fast as nonproducing CHO cells. The latter is true even for the α6-producing clones (CHO-9, 106 and CHO-12, 28), which synthesize up to 3.2×10^4 HU/ml/day (see Table I). The experiments with exogenously added α4 and α6 show a clear IFN dosage effect: a dose of 20 IU/ml α6, corresponding to 40 HU/ml, causes a stronger growth inhibition than a dose of 3,000 IU/ml α4, corresponding to 6 HU/ml. Because HU are defined as antiviral units on hamster cells, this indicates that a strict correlation exists between the antiviral and the antiproliferative activity on hamster cells of both subspecies. Also investigated was the influence of exogenously added IFN on the growth of the producer cell lines, because, as a consequence of low cell density, at the onset of the proliferation experiments only small amounts of IFN were present in the culture medium. A slight inhibitory effect of exogeneously added IFN-α6 on the growth of the producer cell lines was observed (Fig. 2). Inhibition measured at an IFN dose of 100 IU/ml (200 HU/ml) was identical or even smaller than that found with control CHO cells at an IFN concentration of 10 IU/ml. Similar data were
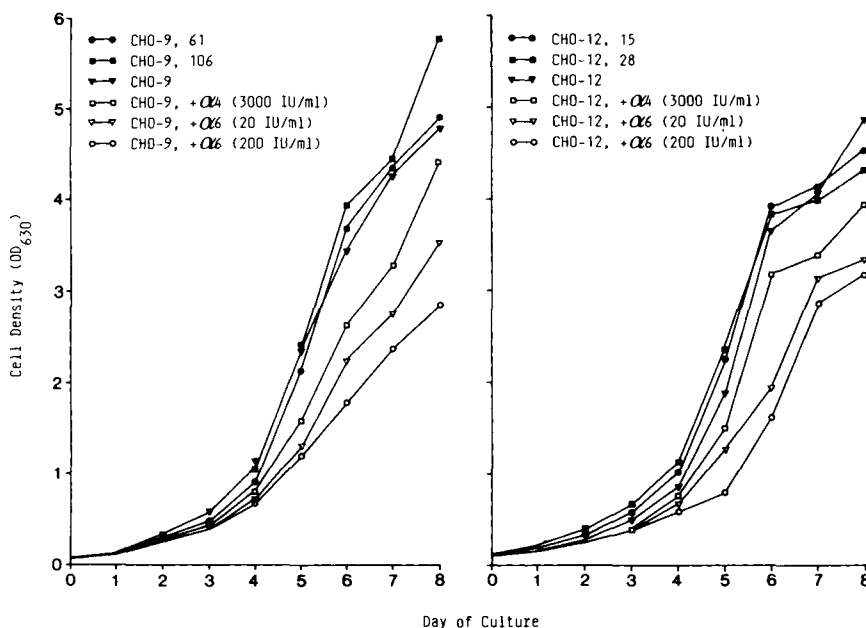

Fig. 1.   Proliferation of CHO cells, CHO cells grown in the presence of Mu IFN-α4 (3,000 IU/ml, corresponding with 6 HU/ml) or -α6 (20 or 200 IU/ml, corresponding with 40 or 400 HU/ml), and IFN-producing CHO cell lines (see Table I for IFN production).
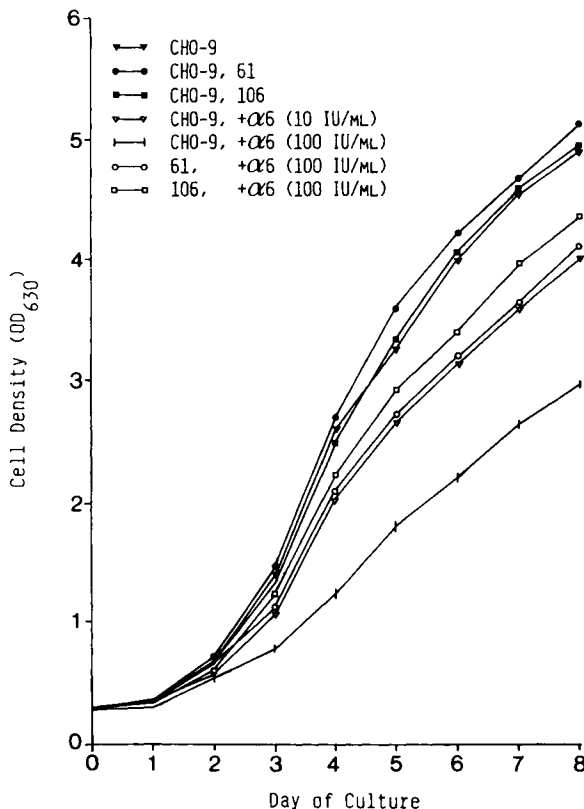
Fig. 2. Proliferation of control and IFN-producing CHO-9 cells in the presence or absence of Mu IFN-α6. Control CHO cells were incubated with 10 or 100 IU/ml α6, corresponding with 20 or 200 HU/ml, and IFN producers with 100 IU/ml α6.

obtained when these experiments were done with the CHO-12 cell lines (results not shown).

All IFN-producer cell lines were analyzed for their ability to support replication of VSV. One day after VSV infection, the control CHO cells were already lysed by the virus, whereas the IFN-producer cell lines were completely protected. This clearly shows that IFN-producer cell lines, including the α4 producers, which secrete only a limited amount of IFN as measured in antiviral units on hamster cells (32–128 HU/ml/day), still respond to the antiviral activity of IFN. In addition, several clones producing smaller amounts of α4 (10–20 HU/ml) were also protected against VSV-induced cell lysis.

Combined, the growth characteristics and the viral protection properties of established CHO-IFN-producer cell lines suggest an uncoupling of the IFN-induced growth inhibition and virus-resistance pathway in this system.

IFN-Induced Gene Expression in CHO Cells

The complete protection against VSV infection shown by IFN-producing CHO cells indicates that the first common step in all biological responses to IFN, which is binding to the specific IFN receptor, is still intact in these cells. However, the present experiments did not exclude modification (decreased binding capacity) or down

regulation of the receptor. Most likely, an uncoupling of antiviral and antiproliferative activity of IFN can be explained by a selective change in IFN-regulated gene expression following long-term exposure to IFN.

A large number of IFN-induced mRNAs have been described in the human [16–19] and in the mouse [20] system. Probes obtained from several laboratories were used to investigate IFN-induced gene expression in hamster cells grown under various conditions. Five of these probes cross-hybridized sufficiently with hamster RNA. The mRNAs detected with the probes representing 1-8, 6-26 [16], ISG 15 (15k) [19], and ISG 54 (54k) [17] proved to be of similar size as mRNAs found in IFN-treated human cells (0.8, 0.8, 0.7, and 2.9 kb, respectively). The 2-5A probe [18] hybridized to a mRNA of about 3 kb in CHO cells.

The 6-26 gene showed a basically high and uninducible expression (data not shown). A similar observation was made by others in Hela cells [21]. Expression of the other four genes (1-8, ISG 15, 2-5A, and ISG 54) was induced in normal CHO cells by IFN treatment for 8, 16, and 24 hours (Fig. 3, lanes 1–7 and 10–12). In general, the level of induced mRNA was higher in $\alpha6$-treated (4 $\times$ 10^3 IU or 8 $\times$ 10^3 HU/ml) than in $\alpha4$-treated cells (16 $\times$ 10^3 IU or 32 HU/ml). This effect was by far the most pronounced for ISG 54 (see Fig. 3, panel D, lanes 1–7 and 11, 12) and hardly detectable for 1-8 expression (Fig. 3A, lanes 1–7 and 11, 12). 2-5A and ISG 15 expression represent intermediate situations in this respect.

Three of the IFN-induced mRNA species (1-8, ISG 15, and 2-5A) were clearly found to be present in the CHO-IFN-producer cell lines (Fig. 3A, B,C, lanes 8, 9, 13, 14). This is the case for both $\alpha4$ (15, 61) and for $\alpha6$ producers (28, 106). In contrast, the ISG 54 transcript was undetectable or present at a very low level in producer cells (Fig. 3D, lanes 8, 9, 13, 14). Exogenously added IFN-$\alpha6$ did not enhance this level (results not shown). These data indicate that 1-8, ISG 15, and 2-5A most probably are not involved in the antiproliferative activity of IFN. On the other hand, the ISG 54k protein seems to be an intriguing candidate for such a role and not essential for the antiviral activity of IFN.

DISCUSSION

This study reports on the construction and properties of CHO cell lines constitutively producing large amounts of the Mu IFN-α subspecies $\alpha4$ and $\alpha6$. The IFN produced by these cell lines is very well suited for further characterization. Earlier, the present authors and others described the application of CHO cells for production of τIFNs from different species [22–24], of human (Hu) IFN-α [25] and -β [26, 27] and Mu IFN-$\alpha1$ [28]. In most of these studies, except for the amount of IFN produced, little attention has been given to other properties of the producer cells. Because τIFNs show no or only minute biological activity on cells of different species [29], the τIFN producers are not affected in a specific way by the IFN-τ protein. However, this probably is not true for Hu α and βIFNs [26, 29, 30]. A Hu IFN-β-producing CHO cell line constructed by McCormick et al. [27] was found to be insensitive to the growth inhibitory activity of IFN, however, in contrast to the present results, also to its antiviral activity, even after adding exogenous IFN-β. This phenomenon was not investigated in more detail. During construction of a Mu IFN-$\alpha1$-CHO-producer cell line, a similar transient growth inhibition was observed as described
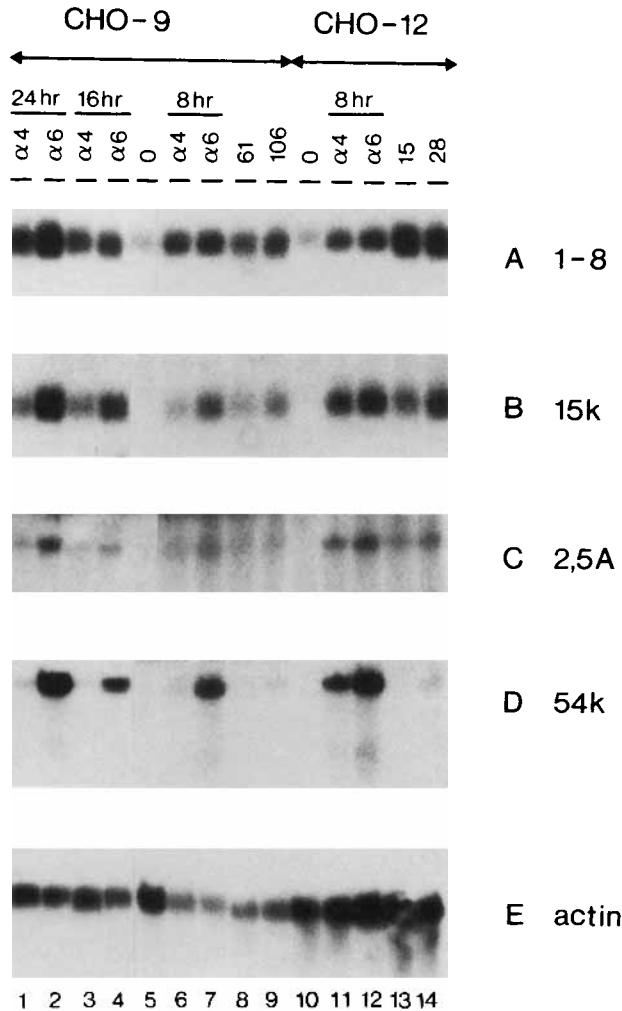
Fig. 3. Northern hybridization analysis of IFN-induced gene expression. The expression of 1-8 (**panel A**), ISG 15 (15k, **panel B**) 2-5A (**panel C**), ISG 54 (54k, **panel D**) and actin (**panel E**) was monitored in CHO-9 and CHO-12 cells (lanes 5 and 10), in CHO-9 cells treated with 1.6×10^4 IU/ml (32 HU/ml) $\alpha4$ or 4×10^3 IU/ml (8×10^3 HU/ml) $\alpha6$ for 8 (lanes 6 and 7), 16 (lanes 3 and 4), and 24 hours (lanes 1 and 2), in CHO-12 cells treated with the same amount of $\alpha4$ and $\alpha6$ for 8 hours (lanes 11 and 12) and in the CHO-producer cell lines 61, 106, 15, and 28 (lanes 8, 9, 13, and 14; see Table I for IFN production).

here for the $\alpha6$ producer [28]. Interestingly, the $\alpha1$ protein also displays high antiviral activity on hamster cells [6].

A method different from the one described here to generate IFN-resistant cell lines is by long-term culture of cells in the presence of increasing amounts of IFN. This method has been applied for establishment of sublines of Daudi [31], L1210 [32], Friend [33], and RSa [34] cells. Most of the resulting cell lines appear to be resistant to both the antiviral and the antiproliferative activity of IFN. In one resistant cell line, L1210, IFN insensitivity is accompanied by a complete loss of functional

IFN receptors from the cell surface [35]. On other resistant cell lines, receptors are present [36, 37], although in some cases a reduction in binding of IFN to its receptor has been observed [38, 39]. Uncoupling of growth inhibitory and antiviral activity has been described for an IFN-resistant subline of Hu RSa cells [34] and for a Hu yolk-sac tumor cell line [40]. Few data are available on specific gene expression in both cell lines. Two enzymatic activities, 2-5A synthetase and 73k protein kinase, are equally well induced in resistant and in sensitive RSa cells [41], whereas in yolk-sac tumor cells 2-5A synthetase, the only enzymatic activity that has been measured, displays a proper induction [40].

Analysis of IFN-induced mRNA expression in a Daudi cell line that is completely resistant to the antiproliferative and partially resistant to the antiviral activity of IFN [42] has revealed three genes (1-8, 6-16, and 9-27) that fail to respond to IFN in the resistant cells, whereas 2-5A synthetase mRNA is induced equally well in resistant and sensitive cells. In the present analysis, 6-16 and 9-27 were not included, because they do not cross-hybridize with hamster RNA. Regarding the 1-8 gene, the present results do not point to involvement of this gene in growth inhibition in CHO cells (see below).

The resistance of the IFN-producing CHO cells to the antigrowth activity of IFN is correlated with a very low level of ISG 54 (54k) transcripts. In contrast, three other IFN-inducible mRNAs (1-8, ISG 15 (15k), and 2-5A synthetase) are clearly present in the producer cells and are not (or hardly) detectable in uninduced cells. This observation suggests that 1-8, ISG 15, and 2-5A synthetase do not have a role in growth inhibition in this system. Moreover, because ISG 54 expression correlates properly with growth inhibition (see Figs. 3D and 1), its gene product is a likely candidate for mediating the growth inhibition induced by IFN. As can be seen from Figure 3A, such a correlation cannot be established for 1-8 expression, as maximal induction of 1-8 already occurs with 32 HU/ml α4, a dose unable to elicit a maximal inhibition of cell growth. A function of ISG 54 in cell-growth regulation might be elucidated by the introduction of the ISG 54 coding region, cloned downstream of a strong constitutive or inducible promoter in, respectively, antisense or sense direction, into the appropriate cells, and determination of the growth characteristics of the resulting cell lines. Although the cDNA has been completely sequenced, so far the properties of the 54k protein are completely unknown. No similarities with other proteins have been observed, except for a small region of 14 amino acids that shows homology to a 13 amino acid stretch present in the mouse Mx protein [43].

To obtain a more detailed insight into the mechanism of resistance to IFN in the CHO producer cell lines, we are presently extending the analysis of IFN modulated genes. Special attention is being given to those genes known to be involved in regulation of cell proliferation and also shown to be modulated by IFN, for example, c-myc [42, 44, 45] or the receptors for certain growth factors, like EGF [46], transferrin [47], and insulin [48].

Another approach for detecting specific genes involved in IFN-mediated growth inhibition can be provided by a comparison of the effects of IFN-α,β with those of IFN-τ on cellular functions. For example, IFN-τ has been shown to restore the antiviral state in IFN-α,β resistant Friend cells, a phenomenon accompanied by an IFN-τ-induced 67k protein kinase activity but not by induction of 2-5A synthetase activity [49]. Unfortunately, the hamster IFN-τ is not available; thus, in this system, these experiments cannot be done as yet.

ACKNOWLEDGMENTS

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