EXHIBIT C

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Inducible Expression of Amplified Human Beta Interferon Genes in CHO Cells

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Plasmid DNA containing the human beta-interferon (IFN-β) gene and mouse dihydrofolate reductase cDNA was transfected into dihydrofolate reductase-negative Chinese hamster ovary cells. Dihydrofolate reductase-positive transformants were obtained, and cells containing amplified copies of mouse dihydrofolate reductase were selected by exposure to increasing methotrexate concentrations. These cells were found to express high levels of human IFN-β after polyriboinosinic acid-polyribocytidylic acid superinduction or NDV infection; this was a result of coamplification of the IFN-β gene. Levels of expression of 1 U/cell per day were achieved on superinduction, giving corresponding titers of up to 10^{10} U/liter medium in culture supernatants. Constitutive production of IFN-β at rates of about 0.5% of superinduced rates was observed; cells producing these levels of IFN-β had acquired resistance to cytotoxic antiviral effects of IFN-β. Two forms of human IFN-β were produced; a major glycosylated 23,000-dalton form and an unglycosylated 18,500-dalton form. The latter had greatly reduced antiviral activity. IFN-β production was very sensitive to cellular growth rate; the highest levels were produced by density-arrested cultures. Regulation of IFN-β production by polyriboinosinic acid-polyribocytidylic acid or by cell density effects required the presence of DNA sequences 5′ to the IFN-β-coding sequences; replacement of these sequences with the simian virus 40 early promoter resulted in uninducible, density-independent production of IFN-β.

Interferons are secreted polypeptides that protect cells from virus infection. Two classes of human interferon (IFN) have been described: type I IFNs (IFN-α, IFN-β) are induced by viral infections and are acid stable and type II IFN (IFN-γ) is induced after stimulation of T-lymphocytes and is acid sensitive. IFNs of both types have been purified to homogeneity, and their biochemical and biological properties have been studied extensively (19, 26). Genes coding for these polypeptides have been cloned, and their nucleotide sequences have been determined (5, 8, 9, 12, 15, 17, 27).

Human IFN-αs comprise a family of at least 12 closely related genes. They are produced by several types of cells, including leukocytes and lymphoblastoid cells, after viral infection. Human IFN-β is secreted by fibroblasts in response to viral infection or exposure to synthetic double-stranded RNA (26). In contrast to the IFNαs, human IFN-β is glycosylated and has an apparent molecular weight of about 22,000 daltons. The single gene encoding IFN-β is expressed in *Escherichia coli* (7, 27). The product of this gene retains the biological activities of its natural counterpart in vitro. However, IFN-β produced in *E. coli* is not glycosylated, and its physical properties and specific activity differ significantly from those of the natural product (Mark et al., submitted for publication). We therefore sought to express the IFN-β gene in a host cell that would provide substantial quantities of glycosylated IFN-β for biological and biochemical characterization. This was achieved by constructing a plasmid containing human DNA encoding the IFN-β gene linked to DNA encoding mouse dihydrofolate reductase (DHFR). This plasmid was introduced into DHFR⁻ CHO cells and cell lines containing multiple copies of the mouse DHFR gene were selected by exposure to increasing concentrations of the DHFR inhibitor methotrexate. These cells were found to contain multiple copies of the IFN-β gene and could be induced to secrete human IFN-β1 at rates up to 300 times those of human fibroblasts. Furthermore, this IFN was glycosylated and had a specific activity of over 10^9 U/mg of IFN protein. An unglycosylated, relatively inactive form of IFN-β was also secreted from these cells. The inducible expression of both of these gene products was found to be a function of cell density and growth rate.

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MATERIALS AND METHODS

Cells. Chinese hamster ovary (CHO) cells and derivatives containing transfected plasmids were grown in Dulbecco modified Eagle medium supplemented with 8% fetal calf serum and 35 μg of proline per ml. DHFR⁻ mutants of CHO cells (29) were grown in Ham F12 medium supplemented with 4% newborn serum and 4% fetal calf serum. All cells were free of mycoplasma contamination.

Plasmids. (i) pMI7. A 1.8-kilobase (kb) *Eco*RI fragment of human DNA containing IFN-β-coding sequences (243 base pairs [bp] of 5′ sequences and 714 bp of 3′ sequences) was ligated into the single *Eco*RI site of pSVM DHFR, a plasmid containing mouse DHFR cDNA 3′ to a mouse mammary tumor virus long terminal repeat and a simian virus 40 (SV40) origin of replication (14). The structure of pMI7 is shown in Fig. 1.

(ii) pGC10. pGC10 contains the coding sequence and 3′ flanking sequences of IFN-β fused to the SV40 early promoter and inserted between the *Bam*HI and *Eco*RI sites of pSVM DHFR. This was accomplished in several steps. First, plasmid pGR1 was constructed by cloning a 340-bp *Bam*HI-adapted *Pvu*II to *Hin*dIII site that encodes the SV40 origin and early promoter between the *Bam*HI and *Hin*dIII sites of pBR322. The structure of pGR1 is shown in Fig. 1. Next, the 838-bp *Taq*I fragment of the IFN-β gene, which

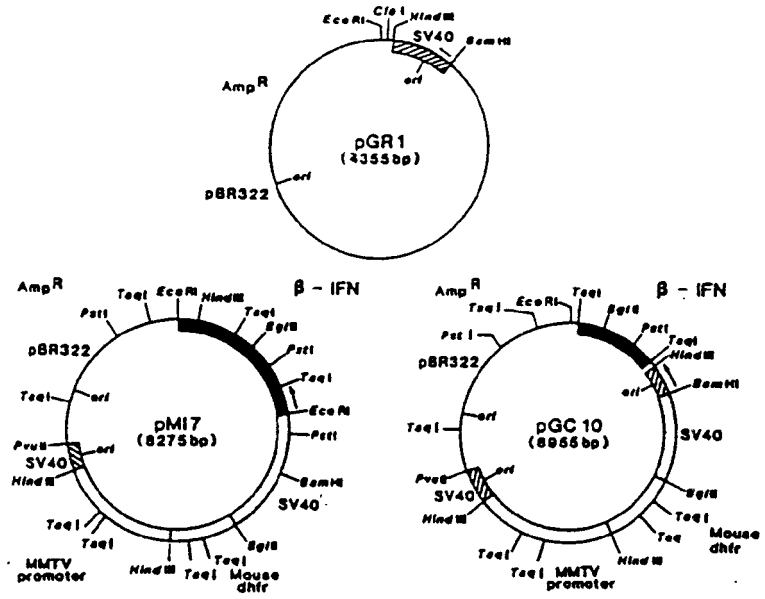

FIG. 1. Plasmids used for expression of human IFN-β in CHO cells.

contains 55 nucleotides of the 5′ untranslated leader, the structural gene including the polyadenylic acid addition signal, and 18 nucleotides of 3′ flanking sequence was isolated by electrophoresis and cloned into the *Cla*I site adjacent to the *Hin*dIII site of pGR1. Recombinant clones in the correct orientation were identified by restriction enzyme analysis. The fused gene was excised by *Eco*RI-*Bam*HI digestion and cloned between the *Bam*HI and *Eco*RI sites of pSVM DHFR. The structure of the resulting plasmid, pGC10, is shown in Fig. 1. Transfection of these plasmids into DHFR$^-$ cells, selection of transformants, and selection of cells able to grow in increasing concentrations of methotrexate (i.e., cells containing multiple copies of the DHFR gene) were performed as described by Ringold et al. (22).

IFN induction and assay. IFN was induced by the addition of polyriboinosinic acid-polyribocytidylic acid [poly(rI-rC)] (20 μg/ml) and cycloheximide (2 μg/ml) to confluent monolayers for 3 h at 37°C. Actinomycin D (2 μg/ml) was added to the cells, and 1 h later they were rinsed in phosphate-buffered saline and given fresh medium. This protocol is similar to that described by Havell and Vilcek (11). IFN activity was assayed by a modification of the method of Finter (6). Samples for assay were diluted to a volume of 75 μl in minimal essential medium, diluted serially in microtiter wells, and sterilized by irradiation with UV light. Approximately 1.2×10^5 human fibroblasts (GM2504) were added to each well, followed by 1 PFU of vesicular stomatitis virus per cell, and the cytopathic effect was scored after 18 to 24 h. The titers were estimated relative to National Institutes of Health IFN standards.

Metabolic labeling of IFN. (i) [^{35}S]methionine labeling. Cells were incubated at 37°C from 4 to 24 h after superinduction in methionine-free medium in the presence of added [^{35}S]methionine (100 μCi/ml, 1164 Ci/mmol; New England Nuclear Corp.).

(ii) [^{14}C]glucosamine labeling. Cells were incubated in complete medium (8% fetal calf serum, Dulbecco modified Eagle medium, proline [35 μg/ml]) in the presence of added DL-[^{14}C]glucosamine (500 μCi/ml, 325 mCi/mmol; New England Nuclear) from 4 to 18 h after superinduction.

(iii) [^{3}H]mannose labeling. Cells were labeled for 30 min 4 h after superinduction in glucose-free medium in the presence of added DL-[^{3}H]mannose (250 μCi/ml, 24.3 Ci/mmol; New England Nuclear). Fresh medium was then added to these cells for the remainder of the superinduction period.

Genomic DNA blot analysis. Southern analysis was performed as described by Stetler et al. (25), except that restricted DNA was transferred to Gene Screen (New England Nuclear), and filters were prehybridized (9 h) and hybridized (72 h) in the absence of dextran sulfate. pMI7 plasmid DNA was labeled by nick translation (21). M13 IFN-β DNA was labeled by the procedure of Hu and Messing (13).

RNA isolation and dot-blots. RNA was prepared from cells lysed in guanidine thiocyanate as described by Chirgwin et al. (4). After centrifugation though CsCl, RNA was suspended in 10 mM Tris (pH 7.4)–1 mM EDTA and ethanol precipitated. RNA was applied to nitrocellulose filters using a Hybri-Dot manifold (Bethesda Research Laboratories, Inc.) in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). After baking for 2 h in vacuo at 80°C, the filter was prehybridized, hybridized, and washed as described previously (3). The probe used was the 1.8-kb *Eco*RI fragment encoding IFN-β nick translated (21) to a specific activity of 10^8 cpm/μg. The filters were exposed to film with an intensifying screen at −70°C for the indicated periods of time.

Immunoprecipitation. Tissue culture supernatants were incubated with 10 μl of anti-IFN-β antiserum per ml. This serum (gift of Leo Lin, Cetus Corp.) was raised by the

injection of 99% pure *E. coli*-produced IFN-β into rabbits. After 1 h at 22°C, 20 μl of protein A-Sepharose (IgGsorb; Enzyme Centre, Inc.) was added. Thirty minutes later, the supernatants were centrifuged (Eppendorf microfuge, 30 s), and the immunocomplexes were washed three times with 0.15 M NaCl–0.5% Nonidet P-40–10 mM Tris (pH 8.0) and eluted with sodium dodecyl sulfate-gel sample buffer as described elsewhere (24).

RESULTS

Levels of IFN expression from transformed CHO cells. The plasmids shown in Fig. 1 were introduced into DHFR⁻ CHO cells, and DHFR⁺ transformants were selected and cloned. Levels of IFN produced constitutively or on induction were determined (Table 1). In each case, the species of IFN produced was identified by its ability to be neutralized by specific antisera. Supernatants from transformed CHO cells were assayed for the presence of hamster IFN by using BHK or CHO cells in the antiviral assay described above. No detectable hamster IFN (i.e., less than 10 U/ml) was produced under the induction conditions described.

Human IFN-β was secreted constitutively from cloned cell lines containing pGC10 (in which the SV40 early promoter is 5′ to the IFN-β1-coding sequence) and from cells containing pMI7, which was expected to transcribe IFN-β mRNA from its own promoter. In the latter case, IFN production was inducible by infection with Newcastle disease virus or by addition of poly(rI-rC) in the presence of cycloheximide (Table 1). Higher levels of IFN were obtained with the superinduction protocol than by Newcastle disease virus infection. Several independent transformants of pMI7 gave similar results.

Derivatives of CHO.MI7 were selected for their ability to grow in the presence of the folate analog methotrexate. Table 1 shows that CHO.MI7 derivatives resistant to 10, 30, and 1000 nM methotrexate (designated CHO.MI7.R10, CHO.MI7.R30, CHO.MI7.R1000, respectively) produce increased levels of IFN constitutively and after poly(rI-rC) superinduction. The rate of production of human IFN-β from CHO.MI7.R1000 corresponds to 1 U/cell per day; this is approximately 300 times higher than rates of production from human fibroblasts induced with a similar protocol (Table 1) (2, 19).

Expression of IFN-β RNA in methotrexate-sensitive and -resistant cells. To assess whether increased production of human interferon was due to overproduction of its corresponding RNA, we determined the relative amounts of IFN-β RNA in CHO.MI7 and CHO.MI7.R30 cells by dot-blot analysis. The basal level of IFN RNA in MI7 is virtually undetectable and can be induced a minimum of 15-fold 12 h after exposure to cycloheximide and poly(rI-rC) (Fig. 2). In the methotrexate-resistant CHO.MI7.R30 cells, there is at least 20 times more IFN RNA produced constitutively, and similar inducibility of IFN mRNA by cycloheximide and poly(rI-rC) is retained. Thus the increased production of IFN-β in the methotrexate-resistant cells is consequent to increased accumulation of IFN RNA.

TABLE 1. Production of human IFN-β from CHO cells[a]

Cell line	IFN produced (U/10⁶ cells per day)		
	Uninduced	Newcastle disease virus induced	Poly (rI-rC) superinduced
CHO.DHFR⁻	0	0	0
CHO.GC10	30	30	30
CHO.MI7	15	3,000	19,000
CHO.MI7.R10	160	1,000	35,000
CHO.MI7.R30	2,000	100,000	475,000
CHO.MI7.R1000	5,000	ND	1,000,000
CTT2	0	1,000	4,000

[a] In all cases except CTT2 (human fibroblasts), cells were seeded from confluent monolayers to a density of 5×10^5 cells per cm^2 24 h before superinduction. IFN-β secreted during this period (uninduced) and 24 h after induction was titered relative to National Institutes of Health IFN-β standards on human GM2504 cells with vesicular stomatitis virus challenge. CTT2 cells were held at confluency for 2 weeks before induction. ND, Not done.

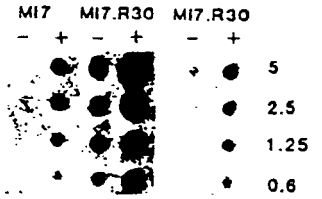

FIG. 2. Dot-blot analysis of RNA from CHO.MI7 and CHO.MI7.R30 cells. RNA was extracted from cells, transferred to nitrocellulose filters, and probed as described in the text. RNA from uninduced (−) and superinduced (+) cells was analyzed. RNA was extracted 12 h after the addition of poly(rI-rC) and cycloheximide and loaded onto filters in the quantities shown. Autoradiography was for 21 days. The right-hand panel (MI7.R30) is a 1-day exposure of the same filter.

Increased expression appears to be a result of gene amplification. Schimke et al. (23) have shown that exposure of cells to a regime of increasing methotrexate leads to amplification of the DHFR gene. This phenomenon is not restricted to the natural gene since recombinant plasmids containing DHFR cDNA can also be amplified (22). Southern blotting analysis was performed on DNA from CHO.DHFR⁻, CHO.MI7, CHO.MI7.R10, CHO.MI7.R30, and CHO.GC10 cells to determine whether gene amplification had occurred in these cells. Figure 3A shows the relative number of *Eco*RI-digested DNA sequences hybridizing to nick translated pMI7. Clearly, this DNA is most abundant in CHO.MI7.R30. Inspection of the autoradiogram suggests that CHO.MI7.R10 and CHO.MI7.R30 contain, respectively, about 5 times and 25 times copies of pMI7 DNA (pMI7 DNA was loaded on the gel at concentrations corresponding to 5 and 25 copies per cell). The sizes of the major *Eco*RI fragments were 17, 13, 6.4, and 2.9 kb. In addition, several minor fragments were detected. The 1.8-kb *Eco*RI fragment that contains the IFN-β gene and flanking sequences was not detected with labeled pMI7 as a probe, implying that the point of integration of the plasmid is somewhere within this fragment. Furthermore, the *Eco*RI digestion pattern of pMI7 sequences indicates that there have been at least two independent integration events in this cell line, or, alternatively, a single integrated plasmid has undergone further rearrangement. These possibilities are suggested by the presence of more than three *Eco*RI fragments that hybridize to pMI7.

The nitrocellulose filter used in Fig. 3A was stripped of hybridizing plasmid DNA and reprobed with a labeled derivative of M13 containing the 1.8-kb IFN-β *Eco*RI fragment. Only the 2.9-kb *Eco*RI fragments and a poorly hybridizing 1.4-kb fragment were detected with this probe.

DNA from CHO.MI7.R30 was subjected to further Southern analysis with *Taq*I and a combination of *Pst*I and *Eco*RI.

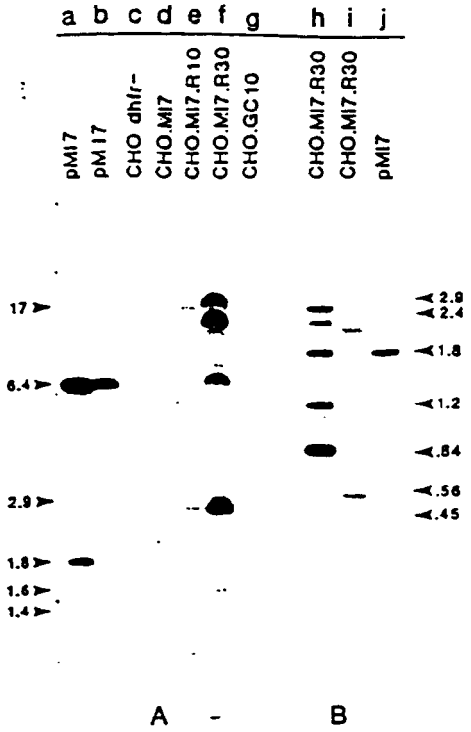

FIG. 3. Southern blot analysis of DNA from CHO lines. DNA was digested to completion with *Eco*RI (lanes a through g and j), *Taq*I (lane h) or *Pst*I-*Eco*RI (lane i). Samples (7 μg) of genomic DNAs were electrophoresed on a 0.6% agarose gel (lanes a through g) or a 1.3% agarose gel (lanes h through j). Samples of 62.5, 92, and 18 pg of *Eco*RI-cut pMI7 DNA were loaded in lanes a, b, and j, respectively. Panel A was probed with ^{32}P-labeled pMI7, and panel B was probed with ^{32}P-labeled human IFN-β in M13.

Restricted DNA was probed with a labeled M13 vector containing the 1.8-kb *Eco*RI fragment, which includes the IFN-β gene (Fig. 1). The entire IFN-β coding sequence and polyadenylation site are contained on a single 840-bp *Taq*I fragment (27); this fragment was shown to be present by this analysis (Fig. 3B, lane h). In addition, a 2.4-kb fragment corresponding to sequences 5′ to IFN-β and including the 5′ end of mouse DHFR was identified. The *Pst*I-*Eco*RI double digest revealed the presence of a 5′ fragment (560 bp) expected to contain the IFN-β promoter as well as fragments of 2.3 and 1.9 kb (Fig. 3B, lane i). Since the 1.24-kb fragment that would contain IFN-β sequences 3′ to the single *Pst*I site was not detected, we conclude that each integration event occurred within this fragment, and that the 2.3- and 1.9-kb fragments contain pMI7-host junctions.

Characterization of human IFN-β produced by CHO.MI7.R30. (i) **Antiviral activity.** IFN-β produced by CHO.MI7.R30 was assayed for antiviral activity on several cell lines. Like authentic IFN-β from human fibroblasts, it was active on GM2504 (a human diploid fibroblast line) and 100-fold less active on BHK, CHO, and MDBK cells (bovine kidney cells, data not shown).

CHO.MI7.R30 cells, which secrete 2,000 U/10^6 cells per day constitutively, were able to support replication of vesicular stomatitis virus even in the presence of 5,000 U of added IFN-β per ml (Table 2). In contrast, replication of vesicular stomatitis virus in CHO.DHFR$^-$ cells was reduced significantly by prior exposure to 5,000 U of IFN-β per ml. This level of human IFN-β also had a cytostatic effect on CHO.DHFR$^-$ cells (A. Creasey, personal communication). CHO.DHFR$^-$ cells produced no detectable hamster IFN (i.e., less than 10 U/ml when titered on BHK-21 cells) under the conditions used for poly(rI-rC) superinduction.

(ii) **Specific activity.** The specific biological activity of IFN-β produced by CHO cells was determined by two methods. First, serum-free medium containing high titers of IFN-β was concentrated by lyophilization and run on sodium dodecyl sulfate-polyacrylamide gels. The amount of IFN-β protein was estimated by Coomassie blue staining and comparison to standards of known concentration. This procedure gave an approximate specific activity of 10^9 U of IFN per mg of IFN protein (data not shown). Second, a metabolic radiolabeling protocol was used: CHO.MI7.R30 cells were superinduced as described above and were labeled for 2 h after superinduction with [^{35}S]methionine in serum-free, methionine-free medium. The amount of [^{35}S]methionine incorporated into IFN-β was determined by running a sample of the supernatant on a sodium dodecyl sulfate gel (Fig. 4, lane B), localizing IFN-β by autoradiography, and measuring radioactivity in the eluted IFN-β band. In one experiment 1.1×10^7 dpm of IFN-β was recovered; this corresponded to 2×10^5 U of IFN-β activity. The specific radioactivity of the methionine in cellular pools was determined by extraction of the cell pellet in 10% trichloroacetic acid followed by amino acid analysis in a Beckman analyzer (K. Watt, Cetus Corp.). The specific radioactivity was found to be 3.56×10^5 dpm/pmol. Assuming that the specific radioactivity of the four methionine residues in IFN-β was equal to the specific radioactivity of methionine in the precursor pool, it can be calculated that the amount of IFN-β produced by these cells was 7.5 pmol, or 1.7×10^{-4} μg (IFN has a molecular mass of 23,000 daltons). The specific biological activity was therefore 1.2×10^9 U/mg of IFN protein.

(iii) **Metabolic labeling of CHO IFN-β.** Monolayers of CHO.MI7.R30 cells were superinduced and labeled with [^{35}S]methionine, and medium from these cultures was immunoprecipitated with anti-IFN-β antiserum. Two polypeptides reacted specifically with this serum (Fig. 4, lane D). The apparent molecular masses of these peptides are 23,000 and 18,500 daltons. Proteins with identical molecular masses were immunoprecipitated specifically from cultures of superinduced human fibroblasts (Fig. 4, lane C). The 23,000 dalton species from CHO.MI7.R30 could also be radiola-

TABLE 2. Virus yields on BHK-21 cells[a]

Cell line	IFN-β (5000 U/ml)	Virus yield (PFU)
CHO.DHFR$^-$	–	1.3×10^8
CHO.DHFR$^-$	+	2.5×10^3
CHO.MI7.R30	–	5.2×10^8
CHO.MI7.R30	+	6.0×10^8
GM 2504	–	3.0×10^8
GM 2504	+	5.6×10^3

[a] Cells were infected with vesicular stomatitis virus at a multiplicity of infection of 10 PFU per cell. Cells were treated with human IFN-β (from CHO.MI7.R30; 5,000 U/ml) for 24 h before infection in Ham F12 medium plus 5% fetal calf serum.

beled with [^{14}C]glucosamine or [^{3}H]mannose (Fig. 4, lane E; Fig. 5). However, these radiolabels were not incorporated into the 18,500 dalton species at detectable levels. In addition, the 23,000 dalton form bound specifically to concanavalin A-Sepharose and was eluted with α-methyl mannoside in ethylene glycol (data not shown). This eluted fraction contained all of the recovered antiviral activity (50% of the input). The 18,500 dalton form flowed through the column. The flow-through fraction contained less than 0.2% of the input antiviral activity. Figure 4 (lane B) shows that IFN-β (apparent molecular mass, 23,000 daltons) comprises about 5% of the proteins secreted by CHO.M17.R30 after poly(rI·rC) superinduction. The identity of the 11,000 dalton polypeptide is not yet known; this polypeptide, like the 23,000 and 18,500 dalton forms of IFN-β, is only detected in supernatants from superinduced cultures.

The 23,000 and 18,500 dalton polypeptides were eluted from sodium dodecyl sulfate-polyacrylamide gels, and antiviral activity was determined. In one experiment (Fig. 5), 3,000 U of IFN activity was recovered from the 23,000 dalton species, and no IFN activity (i.e., less than 10 U) was detected in the 18,500 dalton form.

Effect of cell density on human IFN-β production from CHO cells. Figure 6 shows that the level of interferon produced by cells growing at high density is up to 30 times higher than that produced by sparse cultures when expressed as IFN produced per cell. Cells grown to high density lost their ability to produce high levels of IFN when plated at low density and superinduced within 4 h of plating. However, cells growing at low density did not rapidly acquire the ability to produce IFN when trypsinized and replated to higher density. The highest levels of production

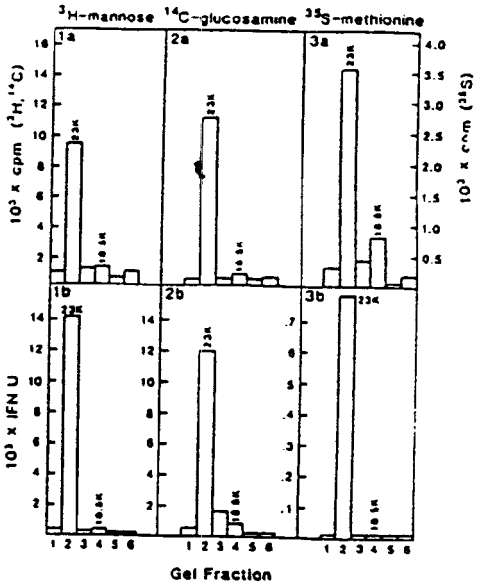

FIG. 5. Antiviral activity and radioactivity associated with IFN-β from CHO.M17.R30 cells. Cells were labeled as described in the text, and supernatants were immunoprecipitated and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as in Fig. 4. Gel fractions were treated with elution buffer overnight, and the eluted radioactivity and antiviral activity were determined.

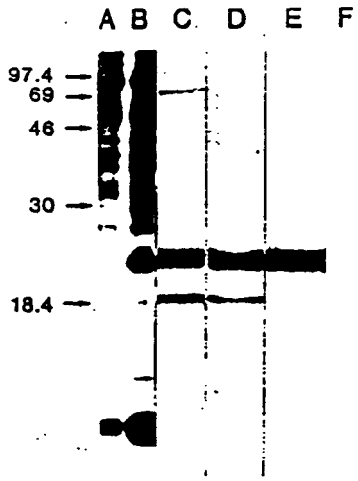

FIG. 4. Metabolic labeling and immunoprecipitation of IFN-β. Cells were labeled with [^{35}S]methionine, 100 μCi/ml, 1,164 Ci/mmol (lanes A through D and F), or [^{14}C]glucosamine, 500 μCi/ml, 325 Ci/mmol (lane E). Lane A contained 10 μl of supernatant from uninduced CHO.M17.R30 cells. Lane B contained 10 μl of supernatant from superinduced CHO.M17.R30 cells. Supernatants from superinduced human fibroblasts (lane C) or CHO.M17.R30 cells (lanes D and E) were immunoprecipitated with rabbit anti-IFN-β antiserum. Lane F contained supernatants from cells immunoprecipitated as in lane D, except that normal rabbit serum was used.

(1 U/cell per 24 h) were achieved when cells were grown to confluence and superinduced 2 or 3 days later. With this protocol, IFN-β titers of 10^{10} U/liter of medium were achieved. These titers were considerably higher than those obtained by induction of the human IFN-β gene integrated in chromosomes of mouse L-cells and rabbit kidney cells (2) or integrated in a bovine papilloma virus vector replicating in C127 mouse fibroblasts (31). This is partly due to the high gene copy number in CHO cells and partly to undefined differences between cell types.

CHO.GC10 cells, which express the IFN-β coding sequences from the SV40 early promoter, produce IFN-β at a constant rate (0.003 U/cell per 24 h) regardless of cell density (data not shown).

DISCUSSION

Expression and regulation of human IFN-β in CHO cells. We have introduced the human IFN-β gene into CHO cells and derived lines that contain multiple copies of this gene by using the coamplication method of Ringold et al. (22). These cells can be induced to secrete human IFN-β at rates up to 300 times greater than human fibroblasts, the conventional source of human IFN-β. Fortuitously, these cells produce undetectable levels of hamster IFN under our induction conditions. Furthermore, cells secreting high levels of IFN-β constitutively had become insensitive to the antiproliferative and antiviral effects of IFN-β. It is not clear whether this insensitivity is the result of a cellular mutation (in the IFN-β receptor, for example), or whether it is the result of a reversible phenotypic change as described in some IFN-resistant cell lines (19).

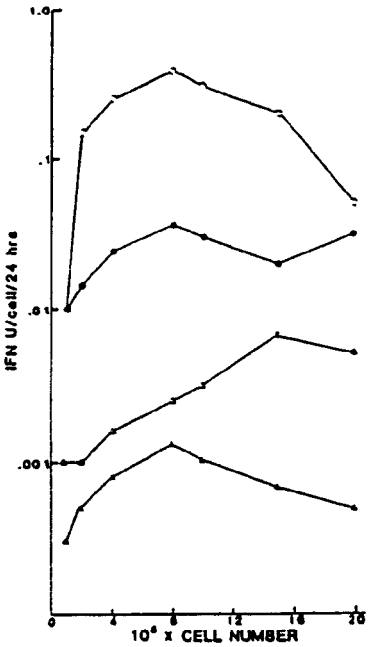

FIG. 6. Effect of cell density on IFN-β production. Cells from sparse cultures or cultures that had been confluent for 2 days were trypsinized and seeded in 2-cm² wells as shown. Four hours later they were superinduced, and the IFN-β titer in the supernatants was determined after 24 h. Symbols: (○) CHO.MI7.R30, confluent; (●) CHO.MI7.R30, subconfluent; (△) CHO.MI7.R10, confluent; (▲) CHO.MI7.R10, subconfluent.

Several points concerning the regulation of IFN-β expression are noteworthy. First, it is clear that the human gene transfected into CHO cells retains its ability to be superinduced. This is consistent with the results of others who have demonstrated inducible expression of known IFN-β in heterologous cells (2, 10, 16, 18, 28, 31). Second, when the SV40 early promoter is fused to the IFN-β coding region, constitutive, but not inducible, expression is observed. The level of expression from the SV40 promoter is not significantly different from the level of constitutive expression for the IFN-β promoter. This is in contrast to reports of others indicating that superinduction of IFN-β is retained by hybrid genes in which the herpes virus thymidine kinase promoter is fused to the IFN-β-coding sequence (20). The reason for this discrepancy is unclear. Third, it is particularly encouraging that normal inducibility of IFN-β expression is maintained in cells containing amplified copies of the gene. The resulting high levels of constitutive and induced expression have enabled us to dissect the mechanisms involved in the superinduction phenomenon (unpublished data). For example, we have been able to show that induction of interferon in CHO.MI7.R30 cells is highly density dependent, and we suggest that cells only produce IFN at high levels in a phase of the cell cycle corresponding to G_0. When cells in this phase are plated at low density, they quickly lose the ability to produce high levels of IFN, presumably because they move out of G_0 toward the S phase. When rapidly growing cells are plated at high density, they gradually acquire the ability to produce high levels of IFN as they accumulate on G_0. These effects on IFN-β expression appear to involve one or more components of the induction pathway. Consistent with this hypothesis, we have observed density-independent IFN-β production from CHO.GC10 cells, in which IFN-β expression is directed by the SV40 early promoter.

Processing and secretion of IFN-β. CHO cells containing human IFN-β DNA secrete two polypeptides that reacted specifically with antiserum raised against *E. coli*-produced IFN-β. These polypeptides have apparent molecular masses of 23,000 and 18,500 daltons and comigrate with polypeptides immunoprecipitated from superinduced human fibroblasts. The 18,500 dalton form also comigrates with IFN-β expressed in *E. coli* (data not shown). The 23,000 dalton form was shown to be glycosylated and highly active (10^9 U/mg of IFN protein). On the other hand, the 18,500 dalton form is not glycosylated and is at least 300 times less active. We do not yet know whether this lack of activity is a direct result of lack of glycosylation. Yip and Vilcek (30) have reported that induction of human fibroblasts in the presence of tunicamycin, which inhibits N-linked glycosylation, resulted in diminished production of IFN activity, and they suggested that this may be a result of altered molecular properties of unglycosylated IFN. However, Bose et al. (1) observed no loss in activity after treatment of IFN-β with glycosidases. Mark et al. (submitted for publication) have reported that IFN-β produced by *E. coli* has a lower specific activity than does authentic IFN-β from human fibroblasts and from these CHO cells, and they suggested that this may be a result of improper sulfydryl cross-linking. It is therefore possible that glycosylation prevents incorrect folding and intramolecular cross-linking and thus confers a higher specific activity. Alternatively, it could be argued that incorrect cross-linking prevents glycosylation. These possibilities are currently being investigated.

In summary, we have established stable cell lines that can be induced to secrete high levels of glycosylated human IFN-β of high specific activity. The availability of these cells will permit us to evaluate the role of glycosylation in the activity of IFN-β and to further study the mechanism of IFN induction.

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