Heterogeneity of Chinese Hamster Ovary Cell-produced Recombinant Murine Interferon-γ*

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Chinese hamster ovary cells transformed with a hybrid expression plasmid containing both the murine interferon-γ (MuIFN-γ) and the murine dihydrofolate reductase-coding sequences were subjected to selection in stepwise increasing concentrations of methotrexate. By this procedure the production rate of MuIFN-γ was increased from an initial level of ~20,000 to ~500,000 antiviral units per milliliter of culture supernatant.

Methionine-labeled proteins secreted by these cells were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with or without prior immunoprecipitation with polyclonal or monoclonal antibodies against splenocyte-derived MuIFN-γ. Besides two major components of $M_r$ 19,000 and 38,000, a multiplicity of minor components were immunoprecipitated. Cells treated with tunicamycin, an inhibitor of N-glycosylation, secrete two major components of $M_r$ 14,000 and 27,000 and only two minor components of $M_r$ 12,000 and 13,000. When the proteins were labeled with [35S]cysteine, a residue that is only present at the carboxyl terminus of the mature MuIFN-γ, no minor components could be detected in the growth medium of tunicamycin-treated cells. The presented results indicate that the heterogeneity of the recombinant Chinese hamster ovary-produced MuIFN-γ is due to at least three cumulative modifications of the $M_r$ 14,000 MuIFN-γ peptide: (i) carboxyl-terminal proteolytic processing (the $M_r$ 13,000 and 12,000 components), (ii) variations in N-glycosylation (components ranging in size from $M_r$ 12,000 to 28,500), and (iii) dimerization (components ranging from $M_r$ 27,000 to 50,000).

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‡The abbreviations used are: MuIFN-γ, murine interferon-γ; CHO, Chinese hamster ovary; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; kb, kilobases; EME, E. coli minimal essential medium; FCS, fetal calf serum.

Gray and Goeddel (3) have described production of biologically active MuIFN-γ by transformed Escherichia coli. We have developed a Chinese hamster ovary (CHO) cell line (DICK-234) that constitutively secretes MuIFN-γ (4). In contrast to the E. coli system, interferon produced by these cells is expected to be processed and glycosylated in much the same way as is the natural molecule.

We now report the selection of several DICK-234 cell sublines which produce MuIFN-γ as a major constituent of their secreted protein pool. These high yields have allowed us to demonstrate a previously unsuspected molecular heterogeneity of MuIFN-γ.

MATERIALS AND METHODS

Selection of Methotrexate-resistant Cell Lines—DICK-234, MICK-1, MICK-2, and MICK-3 were routinely cultured in minimal essential medium-α (Gibco Europe, Paisley, Scotland), supplemented with 5% dialyzed fetal bovine serum.

For methotrexate selection, cells were seeded at $10^6$ cells per tissue culture flask and cultured at $37^\circ$C. Cells were reseeded with minimal essential medium-α without nucleotides supplemented with 5% dialyzed FCS and the appropriate concentration of methotrexate. Cells were further cultured at $37^\circ$C. Medium was replaced every 2nd day in order to remove cell debris and toxic cell metabolites. As soon as single colonies were macroscopically visible, all cell clones were collected and immobilized by trypsinization and inoculated in flat-bottomed microtiter plates at 0.2 cells per cup in medium containing the same concentration of methotrexate as the selection medium. Cells from cups in which only a single colony could be discerned were isolated and expanded for characterization studies and conservation in liquid nitrogen. Methotrexate was omitted from the medium after about five passages.

Interferon Assay—Interferon was titrated in a cytopathic reduction assay on primary mouse embryo cells using mengo virus as the challenging virus (32). Interferon concentrations were expressed as laboratory units.

Southern and Northern Blot Analysis—High molecular weight DNA was isolated as described by Scott et al. (33). Five micrograms of DNA was submitted to electrophoresis in a 0.7% agarose gel with or without prior digestion with a restriction enzyme. HindIII-digested phage λ DNA was used as a molecular weight marker. DNA was transferred to nitrocellulose filters using a blotting procedure (34) and immobilized by baking at 80 °C for 2 h.

Total cytoplasmic RNA was isolated (35) and enriched for poly(A)$^+$ RNA by a batchwise oligo(dT) affinity chromatography essentially as described (36, 37). RNA was denatured with glyoxal and dimethyl sulfoxide (38) and submitted to electrophoresis in a 1% agarose gel (34). E. coli 23 S and 16 S rRNAs (Boehringer Mannheim, West Germany), used as molecular weight markers, were visualized by ethidium bromide staining. RNAs were blotted to nitrocellulose filters (39) and immobilized by baking at 80 °C for 2 h.

For use as a probe in DNA and RNA hybridization experiments, the 0.9-kb Sau3A-SmaI fragment of PM1FK01 (probe A), or the 1.1-kb EcoRI fragment of pAD521 (probe B) (4) were 32P-labeled to high specific activity by nick translation. Probe A comprises the complete coding region and fragments of the noncoding regions of the MuIFN-γ cDNA, whereas probe B, in addition to these sequences, also includes the SV40 origin of replication. Hybridization conditions were as described by Wahl et al. (40) except that dextran sulfate was used.

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omitted from the hybridization mixture.

Cells labeled in the presence of tunicamycin (Sigma) were first preincubated with the antibiotic at 0.01–10 μg/ml for 6 h, followed by labeling with [35S]methionine or [35S]cysteine, also in the presence of tunicamycin (0.01–10 μg/ml).

**Labeling of CHO Cell-secreted Proteins—**Confluent monolayers of cells were refed with methionine-free Eagle's minimal essential medium (EMEM) supplemented with 5% fetal calf serum (FCS) and 50 μCi/ml [35S]methionine (Amersham, Amersham, United Kingdom, 110 Ci/mmol). Alternatively, cysteine-free EMEM supplemented with 5% FCS was refed with methionine-free EMEM supplemented with 5% FCS and 50 μCi/ml [35S]methionine. After 1 h at 37 °C, medium was replaced with normal EMEM supplemented with 5% FCS and cells were further incubated at 37 °C. The supernatant fluid containing the labeled secreted proteins was withdrawn after various time intervals and concentrated as above.

Cells labeled in the presence of tunicamycin (Sigma) were first preincubated with the antibiotic at 0.01–10 μg/ml for 6 h, followed by labeling with [35S]methionine or [35S]cysteine, also in the presence of tunicamycin (0.01–10 μg/ml).

**Labeling of E. coli-derived MuIFN-γ—**The MuIFN-γ cDNA was tagged by placing the λ phage P2 promoter, a ribosome-binding sequence, and an ATG codon directly in front of the putative mature interferon coding sequence (His-Gly-Thr-Val... Ref. 9). E. coli harboring the MuIFN-γ expression plasmid, and a temperature-sensitive phage λ repressor gene were grown in 1 l of LB medium at 30 °C to saturation. The temperature was shifted to 42 °C and 50 μCi/ml [35S]methionine was added. After overnight incubation, cells were recovered by centrifugation (1 min; 10,000 × g) and resuspended in 50 ml of 10% sucrose, 50 mM EDTA, 50 mM Tris-HCl, pH 8.0. After three freezing-thawing cycles, 200 μg of lysozyme and 50 μl of 0.2% Triton X-100, 100 mM EDTA, 50 mM Tris-HCl, pH 8.0, was added. Lysis was allowed to occur at room temperature for 5 min and was followed by centrifugation (10 min; 16,000 × g) in order to remove cell debris and nonlysed cells. The supernatant fluid was concentrated to one-tenth of its original volume and used for immunoprecipitation.

**Immunoprecipitation—Staphylococcus aureus cells of the Cowan I strain wearing a coat of protein A (Pansorbin cells, Behring Diagnostics) were used to aid in immunoprecipitation of complexes formed between MuIFN-γ and antibodies. Prior to use, Pansorbin cells were sedimented by centrifugation (4000 × g, 10 min), resuspended in buffer A (0.5% Triton X-100, 0.15 M NaCl, 5 mM EDTA, 0.02% NaN3, 50 mM Tris-HCl, pH 7.4) and allowed to stand at room temperature for 15 min. Cells were collected by centrifugation, resuspended in buffer A, and again pelleted by centrifugation. Finally, Pansorbin cells were resuspended in buffer A supplemented with 1 mg/ml bovine serum albumin and 2 mM methionine (final concentration, 10% w/v).

For immunoprecipitation, 10-μl aliquots of concentrated medium containing the labeled secreted proteins were mixed with 20 μl of 30 mg/ml bovine serum albumin, 2.5% Nonidet P-40, and 20 μl of Pansorbin cells in order to remove nonspecifically binding material. After 5 min at room temperature, the suspension was centrifuged and the Pansorbin pellets were discarded. The labeled proteins in the supernatant fluid were then reacted with either a rabbit polyclonal antiserum (1) or a rat monoclonal antiserum. For immunoprecipitation purposes, three monoclonal antibody-producing hybridoma clones (F1, F2, F3) were used. Monoclonal antibodies F2 and F3 both have good MuIFN-γ binding and neutralization capacities, whereas F1 has good MuIFN-γ binding but poor neutralization properties. The supernatant fluid containing the labeled proteins was placed on ice, and 10 μl of antiserum was added containing about 100,000 neutralizing units per ml (polyclonal antiserum, F2, F3) or about 10 neutralizing units per ml (F1). One neutralizing unit is defined as the amount of antibody required to neutralize 30 laboratory units of interferon. After a contact of 15 min, 0.2 μg of rabbit anti-rat immunoglobulin (Dakopatts, Copenhagen, Denmark) was added to the 5% FCS and supernatant reaction. Antibody-antigen incubation was then continued for a further 15 min at 0 °C. This secondary antiserum was included in the immunoprecipitation reaction mixture, since the monoclonal antibodies are of subclass IgG3, which has poor protein A-binding capacities (41). Indeed, in case the secondary antiserum was omitted, no immunoprecipitation of labeled proteins could be shown using the monoclonal antibodies F1, F2, and F3, whereas using the polyclonal anti-MuIFN-γ antiserum, the secondary antiserum had no effect. Next, 10 μl of Pansorbin suspension was added, the mixture was further incubated at 0 °C for 15 min and centrifuged for 1 min at 8000 × g. Pansorbin cells were washed three times with 0.5 ml of buffer A supplemented with the secondary antiserum and gently resuspended in buffer A supplemented with 2.5% Nonidet P-40 (final concentration). Cells were resuspended in 10 mM Tris-HCl, pH 7.4, 1% SDS, and samples were boiled for 3 min either with (reducing conditions) or without (nonreducing conditions) prior addition of β-mercaptoethanol at 1%. Pansorbin cells were removed by centrifugation, and supernatant fluids containing the immunoprecipitated proteins were either used directly or stored at −20 °C.

**RESULTS**

**Amplification of the MuIFN-γ Production by Transformed CHO Cells—**As a parent line for the amplification of MuIFN-γ production we used DICK-234 cells (4), which are transformed with a plasmid (pADSIF1) comprising the MuIFN-γ coding sequences. For this purpose, methotrexate, a specific inhibitor of dihydrofolate reductase, was included in the growth medium (5). In a first stage, DICK-234 cells were adapted to relatively low concentrations of methotrexate by growing them for 1 month in medium supplemented with gradually increasing sublethal concentrations of methotrexate (2-20 nM). Under these conditions, cell growth decreased but cell death was only marginal. These low methotrexate-resistant cells were then seeded in tissue culture flasks containing growth medium to which 60 nM methotrexate had been added. Single colonies were macroscopically visible after approximately 1 month. The culture was trypsinized and part of the cells was cloned by limit dilution, resulting in the isolation of a representative sub-line (MICK-1). The remainder of the cell suspension was seeded in a tissue culture flask and expanded to 500 nM methotrexate. A clone was selected, although this concentration was then isolated in a similar fashion (MICK-2). Cells were further treated with 3 μM methotrexate, again followed by the isolation of a resistant clone (MICK-3). The selection procedure took 200 days in total.

Methotrexate-resistant cell lines (MICK-1, -2, and -3) were grown to confluency and supernatant fluid was assayed for interferon activity (Table I). The data show that each amplification step resulted in a 3-fold increase in MuIFN-γ production. Interferon production remained constant for at least 1 month.
Fig. 1. Structure of the MuIFN-γ expression plasmid (pADSIF1). Construction of pADSIF1 was described in a previous paper (4). Probe A is the 0.9-kb Sau3A-SmaI fragment of pMIFK01, probe B is the 1.1-kb EcoRI fragment of pADSIF1. → MuIFN-γ sequences; (coding/noncoding); ε, rabbit β-globin sequences; δ, SV40 sequences; φ, adenovirus sequences; γ, dihydrofolate reductase sequences; →, pBR322 sequences; ω, promoter sequences; ••, intron sequences; pA, polyadenylation signal sequence.

TABLE I

**MuIFN-γ production of methotrexate-resistant CHO cell lines**

Cells were grown onto confluency in minimal essential medium-α with nucleotides and without methotrexate. Fresh medium was added and replaced after 48 h for interferon assay. Interferon concentrations are expressed in laboratory units/ml. Values are results of a typical experiment.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Methotrexate resistance</th>
<th>MuIFN-γ production</th>
</tr>
</thead>
<tbody>
<tr>
<td>DICK-234</td>
<td>&lt;2</td>
<td>17,000</td>
</tr>
<tr>
<td>MICK-1</td>
<td>60</td>
<td>58,000</td>
</tr>
<tr>
<td>MICK-2</td>
<td>500</td>
<td>160,000</td>
</tr>
<tr>
<td>MICK-3</td>
<td>3,000</td>
<td>570,000</td>
</tr>
</tbody>
</table>

10 months (55 passages), even when methotrexate was omitted from the medium. Cells that were most resistant to methotrexate had an altered morphology (larger cells) and a longer generation time than the original CHO cells (passaged once a week½ instead of twice a week½).

**Determination of the Level of DNA Amplification**—Total cellular DNA of the cells to be examined (CHO, DICK-234, MICK-1, MICK-2, MICK-3, and L929) was isolated and digested to completion with EcoRI and HindIII. The reaction products were separated by electrophoresis and transferred to filters which were hybridized with a nick-translated DNA probe comprising the MuIFN-γ coding sequences (probe A, Fig. 2). In all CHO cell lines transformed with the MuIFN-γ cDNA, a 4.3-kb HindIII fragment as well as 1.1 and 0.9 kb EcoRI fragments were found. Digestion of the original expression plasmid (pADSIF1) with EcoRI and HindIII followed by Southern blot hybridization of the reaction products gave rise to positive hybridization fragments of apparently identical length (see Fig. 1). Therefore, the data are in agreement with an integration of the complete MuIFN-γ expression unit in the host cell chromosome without major DNA rearrangements.

Comparison of the hybridization intensities of the DNA from the different cell lines by densitometric plotting of the autoradiography allowed us to estimate relative copy numbers of the integrated MuIFN-γ cDNA: MICK-1, -2, and -3 cells contained, respectively, about 8, 30, and 60 times as many copies as the original DICK-234 cell. The absolute copy number in DICK-234 cells is difficult to estimate by this kind of comparison (6). However, comparison with the hybridization signal obtained from murine L929 cells, which are known to contain a single MuIFN-γ gene copy per haploid genome (7) revealed that the parent DICK-234 have only few MuIFN-γ gene copies per diploid genome. Hybridization of the nitrocellulose filter with probe B which, besides the MuIFN-γ coding sequences also includes the SV40 origin of replication sequences, suggested that the MuIFN-γ expression unit may be integrated only once in the genome of DICK-234 cells, since only a single DNA fragment spanning the junction between the plasmid DNA and the host cell DNA was shown (the 2.2-kb EcoRI fragment, Fig. 2).

When the hybridization and washes were performed under less stringent conditions, an additional hybridization signal was observed in lanes loaded with DNA from transformed as well as nontransformed CHO cells (results not shown). This supplementary band probably represents the endogenous hamster IFN-γ gene. A blotting experiment with uncleaved cellular DNA demonstrated that all sequences hybridizing to the MuIFN-γ cDNA were associated with high molecular weight DNA (>100 kb).

**Characterization of the MuIFN-γ RNA in CHO Cells**—Poly(A)+ RNA isolated from CHO, DICK-234, and MICK-3 cells was analyzed by Northern blotting. Samples of the RNAs were electrophoresed in a 1.0% agarose gel and transferred from the gel onto a nitrocellulose filter. 16 S and 23 S E. coli rRNAs were used as molecular weight marker molecules. For hybridization, a nick-translated 0.9-kb Sau3A-SmaI fragment of the MuIFN-γ cDNA (probe A), comprising the complete

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Fig. 2. Southern analysis of MuIFN-γ-coding DNA isolated from MuIFN-γ-producing CHO cells. High molecular weight DNA isolated from DICK-234 cells (lanes 1 and 8), MICK-1 cells (lanes 2 and 9), MICK-2 cells (lanes 3), MICK-3 cells (lanes 4), CHO cells (lanes 5), murine L929 cells (lanes 6), and human Hep-2 cells (lanes 7) was digested to completion with HindIII or EcoRI and submitted to electrophoresis in a 0.7% agarose gel. DNA fragments were transferred to a nitrocellulose filter, immobilized, and hybridized to probe A that had been labeled to a specific activity of $20 \times 10^6$ cpm/μg by nick translation (lanes 1–7). Hybridization and washing conditions were as described under "Materials and Methods." Autoradiography was for 1 week at −70 °C. After removal of the probe, the filter was re-hybridized with probe B (nick-translated to a specific activity of $15 \times 10^6$ cpm/μg; lanes 8 and 9). Autoradiography was for 3 weeks at −70 °C. HindIII-digested phage λ DNA was used as a reference.
MuIFN-γ coding region, was used as a radioactive probe (Fig. 3). The autoradiographs of the hybridized filters revealed the presence of a single hybridizing RNA species in DICK-234 cells, whereas in MICK-3 cells two RNA species were shown. The hybridization intensity of the latter RNAs was about 20-fold that of DICK-234. In untransformed CHO cells no RNAs could be detected that hybridized either to the sense or anti-sense strand of the MuIFN-γ cDNA. The RNAs observed in MICK-3 and DICK-234 cells migrate at 10–15 S, a value in good agreement with a mRNA that is initiated, spliced, and terminated as expected from the expression plasmid construction.

**Molecular Weight Determination of CHO-derived MuIFN-γ**—The CHO cell-produced MuIFN-γ was characterized by SDS-PAGE analysis of secreted [35S]methionine-labeled proteins. For this purpose confluent monolayers of CHO, DICK-234, MICK-1, and MICK-3 cells were refed in “cold” methionine-free medium supplemented with 2% FCS and 50 μCi/ml [35S]methionine. After 16-h incubation, the medium was withdrawn and concentrated by lyophilization. Aliquots were analyzed by SDS-PAGE in the presence of β-mercaptoethanol (reducing conditions) without further purification. An intense protein band corresponding to a molecular weight of about 19,000 was seen in the case of MICK-3 cells, but not in the case of untransformed CHO cells (Fig. 4). In order to demonstrate that this protein band actually co-migrated with MuIFN-γ biological activity, SDS-PAGE gels loaded with similarly obtained but nonradioactive culture fluids of MICK-3 cells were fragmented, and each slice was eluted by incubation in medium supplemented with 5% FCS. The eluted material was then tested for antiviral activity in a cytopathic reduction assay. As expected, a major peak of biological activity was observed in slices corresponding to M, 19,000; minor peaks were at M, 38,000, 25,000, 17,000, and 14,000 (Fig. 4).

The CHO-produced MuIFN-γ was further characterized by immunoprecipitation. [35S]methionine-labeled proteins were reacted with specific antibodies, and the precipitates were analyzed by SDS-PAGE. Proteins immunoprecipitated by a purified polyclonal antibody (1) resolved into several bands.
proteins. A pulse-chase experiment revealed that the ratio of proteins were analyzed by immunoprecipitation with monoclonal antibodies used. In reducing conditions more than 90% of precipitate appeared at a M, value of approximately 19,000. In some preparations the major 19,000 band appeared as a doublet. In all cases, however, multiple minor bands with M, values slightly higher or lower than 19,000 (range 18,000–21,000) were visible. Outside the main M, 19,000 area discrete bands were observed at M, 14,000 and 17,000 as well as a group of bands at M, 23,500. SDS-PAGE analysis in the absence of β-mercaptoethanol (nonreducing conditions) revealed a supplementary major band at M, 38,000 with heterogeneity in that region similar to that seen for the M, 19,000 proteins. A pulse-chase experiment revealed that the ratio of minor/major components remained constant in time and that the M, values of all observed MuIFN-γ-related proteins were identical after a 1-h or a 24-h chase (results not shown).

An increase in intensity of all bands was observed in the amplified CHO cells as compared with the DICK-234 cells, in agreement with the higher levels of antiviral activity observed earlier. Densitometric plotting of the autoradiography revealed that MICK-1, MICK-2, and MICK-3 secrete, respectively, 12, 20, and 60 times as many immunoprecipitable proteins of M, 19,000 as DICK-234 cells.

Glycoprotein Nature of CHO-derived MuIFN-γ—In order to analyze the glycoprotein nature of the CHO cell-produced MuIFN-γ, confluent monolayer cultures of MICK-3 cells were refed with medium supplemented with [35S]methionine and increasing concentrations of tunicamycin, an inhibitor of N-glycosylation (8). Labeled proteins secreted by these cells were used for immunoprecipitation and SDS-PAGE analysis (Fig. 6). Tunicamycin treatment resulted in an altered profile on SDS-PAGE: besides lower M, values of the immunoprecipitable bands, a decreased heterogeneity was also observed.

Instead of at least 10 bands in normal conditions only three discrete bands appeared, corresponding to M, values of 14,000 (~90%), 13,000, and 12,000 in reducing conditions. In nonreducing conditions a supplementary protein band appeared at M, 27,000 (Fig. 7, lanes 1 and 2). These values were compared with those of E. coli-produced MuIFN-γ. Here, immunoprecipitation with monoclonal antibody followed by SDS-PAGE showed a major protein band at M, 13,500 in the presence of β-mercaptoethanol. A minor band was visible at M, 13,000.

Analysis of the Carboxyl-terminal End of CHO-produced MuIFN-γ—Since the putative mature MuIFN-γ molecule contains only a single cysteine residue (9) as the carboxyl-terminal amino acid, its presence could be used as a criterion for the integrity of the MuIFN-γ carboxyl-terminal end. Parallel MICK-3 cell cultures were labeled with [35S]methionine (50 µCi/ml) and [35S]cysteine (50 µCi/ml), either with or without tunicamycin in the medium. Labeled secreted proteins were analyzed by immunoprecipitation with monoclonal antibodies (Fig. 7). The SDS-PAGE profile of cysteine-labeled proteins only differed from that of methionine-labeled proteins in that neither the M, 17,000 [35S]-labeled protein, observed in the absence of tunicamycin, nor the M, 13,000 and 12,000 [35S]-labeled proteins secreted by tunicamycin-treated cells were visible. Accordingly, these proteins can be postulated to lack cysteine residues, implying that they have shortened carboxyl-terminal ends.

DISCUSSION

DICK-234, a CHO cell line that previously had been transformed with the MuIFN-γ cDNA (4) was subjected to three consecutive amplification steps by growth in methotrexate. This resulted in a cell line (MICK-3) that secreted approximately 30 times as much MuIFN-γ as the original line. Also, SDS-PAGE analysis of total [35S]methionine-labeled protein secreted by MICK-3 monolayer cultures showed a major protein band of M, 19,000 that co-migrated with MuIFN-γ bioactivity. This band was below detection level in gels from culture medium of the original DICK-234 cell line. The high interferon production level of MICK-3 cells (about 5 × 10⁶ units/ml/day) remained constant during at least 55 passages in nucleotide-free medium, even if methotrexate was omitted from the culture fluid. On comparing the level of MuIFN-γ-coding RNA in DICK-234 and MICK-3 cells, an amplification of approximately 20-fold was observed. The interferon-coding DNA sequences were amplified about 60-fold and may be located on homogeneously staining regions, rather than on minute chromosomes (10), given the unaltered MuIFN-γ production despite numerous passages in the absence of methotrexate.

The MICK-3 cell line was not only suitable for the production of large amounts of recombinant MuIFN-γ, but also proved to be an excellent tool for the molecular characteri-
prior immunoprecipitation, probably since their concentration is too low to discriminate them from the authentic CHO cell proteins.

Several lines of evidence indicate that MuIFN-γ produced by eukaryotic cells is glycosylated (3, 4, 11, 14). Part of the heterogeneity may therefore be due to variations in extent of glycosylation. We did indeed find that the pattern of immunoprecipitable bands was drastically simplified when tunicamycin, an inhibitor of N-glycosylation (8), was included in the medium of MICK-3 cells during protein synthesis. The major components then appeared with $M_v$ values of 27,000 (dimer) and 14,000 (monomer), probably representing the carbohydrate-depleted $M_v$ 38,000 and 19,000 components observed in the absence of the glycosylation inhibitor. On the basis of two N-glycosylation sites per MuIFN-γ monomer, the differences in $M_v$ values recorded in the presence and absence of tunicamycin would give an estimate of $M_v = 2,500$ average for each carbohydrate chain. This value is not uncommon for asparagine-linked oligosaccharides (15). Protein bands corresponding to $M_v$ 27,000 and 14,000 were also seen as minor MuIFN-γ components in the case of MICK-3 cells not treated with tunicamycin, suggesting that unglycosylated proteins may be normal constituents (~1%) of the CHO-produced MuIFN-γ pool. The discrepancy between the apparent $M_v$, value of the carbohydrate-depleted mature MuIFN-γ monomer as shown by SDS-PAGE ($M_v$, 14,000) and the $M_v$ value of 15,525 predicted by sequence analysis of the MuIFN-γ cDNA (3, 9) might be due to factors other than molecular weight that affect migration rates of proteins on SDS-PAGE. This is illustrated by the observation that the carbohydrate-depleted MuIFN-γ monomer migrated slightly faster than lysozyme ($M_v$, 14,500) but markedly slower than $\alpha$-lactalbumin ($M_v$, 14,400). The major component of MuIFN-γ produced by E. coli migrated slightly faster ($M_v$, 13,500) than the major monomeric MuIFN-γ component secreted by tunicamycin-treated MICK-3 cells. This small difference in $M_v$ value may be due to the possible presence of O-linked carbohydrate chains, whose attachment is known to be unaffected by tunicamycin.

The effect of tunicamycin on the minor bands consisted in a reduction in number and a downward shift in $M_v$ value. Only two minor bands of $M_v$, 12,000 and 13,000 remained. With a carbohydrate moiety of $M_v$, 5,000 (as calculated for the major component) these values would correspond to N-glycosylated counterparts of $M_v$, 17,000 and 18,000. Indeed, protein bands corresponding to these $M_v$ values were seen on SDS-PAGE analysis of immunoprecipitates from MICK-3 cells that had not been treated with tunicamycin.

In contrast to what was seen with the major components, no bands of twice the molecular weight of the minor components were detectable, suggesting that the latter were unable to form dimers. This, together with their smaller $M_v$ value, suggested the possibility that they lacked a carboxyl-terminal fragment containing the unique and terminal cysteine residue (9). This hypothesis was confirmed by the observation that the smaller components became undetectable when the cells were labeled with [35S]cysteine instead of [35S]methionine. The autoradiographic intensity of the methionine-labeled major MuIFN-γ components was approximately twice that of the corresponding cysteine-labeled proteins although both amino acids were added at about the same specific activity. This observation is in agreement with a protein possessing 2 methionine residues and a single cysteine residue, as postulated for mature MuIFN-γ (9). SDS-PAGE of proteins secreted by MICK-3 cells not treated with tunicamycin showed a $M_v$, 17,000 protein that could be labeled with [35S]methionine.

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but not with [35S]cysteine, reinforcing our suggestion that this protein is the glycosylated counterpart of the M, 12,000 carbohydrate-depleted component.

In conclusion, MICK-3 cells can be said to produce three molecular forms of MuIFN-γ monomers, which, without N-glycosylation, have M, values of 12,000, 13,000, and 14,000. Only the M, 14,000 form has the ability to dimerize by its carboxyl-terminal cysteine. The two other forms are smaller because they lack a carboxyl-terminal fragment, comprising at least the carboxyl-terminal cysteine, but also in all probability an additional number of other amino acids. We cannot exclude the remote possibility that loss of carboxyl-terminal amine acids is only partially responsible for their smaller M, value, the other part having to be accounted for by the loss of NH2-terminal amino acids or O-linked carbohydrates. The relatively limited heterogeneity caused by peptide length polymorphism is further compounded by dimerization of the M, 14,000 component and by N-glycosylation of all forms.

Basically, three hypotheses can explain the presence of shortened MuIFN-γ molecules in the medium of transformed CHO cells. A first hypothesis calls for a mutation in the coding region of one or more copies of the MuIFN-γ cDNA that are integrated in the genome of the CHO cells. Mutant genes might have arisen during transfection (16) or amplification procedures (e.g. See Refs. 17–19). However, several arguments argue against this hypothesis. First, mutations occurring during the sequential amplification steps are unlikely to be responsible for this heterogeneity since (i) a multiplicity of immunoprecipitable proteins was also observed in the medium of the parent DICK-234 cells; (ii) no major DNA rearrangements in the MuIFN-γ expression unit could be detected by Southern analysis of genomic DNA prepared from DICK-234 and MICK cells; and (iii) MICK-1, MICK-2, and MICK-3 probably are derived from different low methotrexate-resistant cell clones, yet showed a highly similar SDS-PAGE pattern of immunoprecipitated proteins. Second, alterations in the MuIFN-γ coding sequences that might be generated during the transfection procedure and that therefore might already be present in DICK-234 cells before amplification are improbable since (i) DICK-234 cells only have a single or very few copies of the MuIFN-γ gene, (ii) as few as 1% of genes transferred in mammalian cells may contain some detectable mutations (16), and (iii) DICK-234 were carefully selected from more than 50 MuIFN-γ-producing CHO clones for their highest production levels, diminishing the chance of mutations that negatively affect the antiviral activity of MuIFN-γ.

A second hypothesis that explains the presence of truncated MuIFN-γ molecules could be that the mature MuIFN-γ molecules secreted in the medium of the CHO cells, are degraded by serum- or CHO cell-derived proteases. This possibility seems unlikely, since in a pulse-chase experiment it was found that the ratio of the different MuIFN-γ component in the medium of CHO cells remained constant over a period of 24 h.

A third hypothesis, which we assume to be the most plausible one, is that shortened MuIFN-γ molecules arise by post-translational proteolytic processing before, during, or directly after secretion. This hypothesis is in line with the observation of multiple carboxyl termini in natural IFN-γ from human origin (20) and also with the evidence for natural MuIFN-γ molecules to undergo proteolytic processing (21).

Molecular heterogeneity of IFN-γ obtained either from natural producer cells (e.g. See Refs. 12, 13, 22–27) or from mammalian cells transformed with the IFN-γ gene (4, 28–30) has been reported. This heterogeneity was suggested to be caused by aggregation (4, 13, 24), variations in glycosylation (20, 31), and differences in proteolytic processing (20, 21). Ours is the first report documenting an unexpectedly pronounced heterogeneity of IFN-γ produced by recombinant DNA technology. This heterogeneity could be visualized thanks to the high MuIFN-γ-producing capacity of the methotrexate-resistant transformed CHO cells (MICK-3) and to the availability of highly specific (monoclonal) antibodies against MuIFN-γ. The lower degree of heterogeneity observed earlier by other workers may also have been due to a loss of several minor components during the purification procedure.

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