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Production of a Functional Mouse Interferon γ from Recombinant *Saccharomyces cerevisiae*

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Abstract The mouse interferon gene (*MuIFN-γ*) was cloned and then used to transform *Saccharomyces cerevisiae*. Expressed *MuIFN-γ* protein (MuIFN-γ) was successfully secreted into culture medium due to the presence of the signal peptide of rice amylase 1A. Two different promoters fused to *MuIFN-γ* were tested: glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter and a yeast hybrid *ADH2-GPD* (AG) promoter consisting of alcohol dehydrogenase II (*ADH2*) and *GPD* promoter. Using the hybrid promoter, the accumulation of *MuIFN-γ* transcript was the highest after 24 h cultivation, and then gradually decreased as the cultivation proceeded. However, both cell growth and recombinant MuIFN-γ production reached their peaks after the 4-day cultivation. It was possible to produce 6.5 mg/l of MuIFN-γ without any changes in cell growth. Using *GPD* promoter, the *MuIFN-γ* transcript accumulation and the recombinant MuIFN-γ production followed the same pattern as the cell growth. However, compared to that of the hybrid promoter, the production of recombinant MuIFN-γ was 0.2 mg/l. The secreted MuIFN-γ had estimated molecular masses of 21 kDa and 23 kDa, which were larger than that of the encoded size due to glycosylation. The protection assay against the viral infection indicated that the recombinant MuIFN-γ was bioactive.

Key words: Mouse interferon γ, *Saccharomyces cerevisiae*, hybrid promoter

Interferons are cytokines that play a complex and central role in the resistance of mammalian hosts to pathogens. Interferon γ (IFN-γ) is produced by thymus-derived (T) cells under certain conditions of activation and by natural killer (NK) cells [5]. Besides its antiviral activity, IFN-γ has now been reported as a pleiotropic cytokine involved in several immune responses, including the stimulation of bacteriocidal activity in phagocytes, the stimulation of antigen presentation through class I and class II major histocompatibility complex (MHC) molecules, the orchestration of leukocyte-endothelium interactions, and effects on cell proliferation and apoptosis, as well as the stimulation and repression of a variety of genes whose functional significance remains obscure. Thus, the clinical applications of IFN-γ most often suggested are as anti-tumor and anti-inflammatory agents [1].

Bioactive IFN-γ is in the form of a homodimer without covalent bonds. Several genes for IFN-γ have been cloned from various sources and heterologously expressed in other organisms, including *Escherichia coli*, *Saccharomyces cerevisiae*, and Chinese hamster ovary (CHO) cells [7, 8, 26]. Though there is significant homology of IFN-γ among different species, IFN-γ is known to be species-specific in that mouse IFN-γ (MuIFN-γ) does not show cross-reactivity with other IFN-γ's from different sources [22]. Consequently, MuIFN-γ becomes necessary for the study of potential immunoregulatory agents containing antitumor or antiviral activity using a murine system, which has been best characterized and used as one of the most common animal model systems. The MuIFN-γ consists of 155 amino acid residues, including a signal peptide of 19 residues, and the estimated values of the size of natural MuIFN-γ range from 38 kDa to 80 kDa due to glycosylation and aggregation [11]. Although mammalian cell lines have been transfected to secrete bioactive MuIFN-γ, it is neither easy nor economical to produce a large quantity of bioactive MuIFN-γ [8]. The sonicated extract of MuIFN-γ-transformed *E. coli* showed antiviral activity but no further studies on the most efficient means of production and purification were conducted [11]. In addition, several studies have shown that the specific activity of recombinant cytokine

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expressed using a eukaryotic expression system was superior to that of *E. coli* [15, 17, 18]. Thus, there is still an increasing need to produce a large quantity of bioactive MuIFN-γ in a eukaryotic expression system for biological studies of MuIFN-γ actions; e.g., for clinical applications as well as molecular and immunological characterizations.

Expression of foreign proteins in yeast is known to have advantages over other expression systems in that it is a small eukaryotic GRAS (Generally Recognized As Safe) organism with a long history of applications, and the secretion of foreign proteins can be achieved by fusing the leader peptide to the foreign gene [3, 13, 14]. Secretion of foreign proteins from eukaryotes is preferable to expression in *E. coli*, since in *E. coli*, foreign proteins accumulate in inclusion bodies and the extracellular form of protein product is easier to purify than that of the intracellular one. In this study, the coding sequence for mature MuIFN-γ was introduced and expressed in *S. cerevisiae*. The protein product was derived in order to secrete and its bioactivity was measured using the culture filtrate.

MATERIALS AND METHODS

Strains and Culture Conditions

Plasmids were maintained and propagated in *E. coli* HB101 or DH5α as described in Sambrook *et al.* [24]. The mitogen-stimulated mouse splenocytes were used for the amplification of cDNA encoding MuIFN-γ [11], and *S. cerevisiae* 2805 (*MATα pep4::HIS3 prb1-δ Can1 GAL2 his3 ura3-52*) [25] was used for the heterologous production of MuIFN-γ.

S. cerevisiae was maintained in YEPD medium (1% yeast extract, 2% peptone, and 2% dextrose), and uracil deficient selective (ura^-) medium (0.67% yeast nitrogen base without amino acids, 0.003% adenine and tryptophan, 0.5% casamino acid, 2% dextrose, and 2% agar) was used for the screening of the transformants at 30°C. Primary culture was prepared by inoculating a well-grown single colony on ura^- plate into a test tube containing either ura^- or YEPD broth and incubating for 12 h at 30°C while agitating vigorously (300 rpm). A total of 2×10^8 cells from the primary culture were then transferred to a 300-ml Erlenmeyer flask containing 40 ml of an appropriate expression medium. Since the upstream activating sequence (UAS) of a yeast hybrid (AG) promoter was known to cause repression as well as derepression by the presence of an excess amount of glucose and ethanol, respectively, and a 1.5% (v/v) ethanol supplement gave the best results for the production of foreign protein in our previous study [21], YEP-EtOH (YEP containing 1.5% ethanol) medium was used for AG promoter as an expression medium. YEPD medium was used for *GPD* (glyceraldehyde-3-phosphate dehydrogenase) promoter as described previously [19]. Expression cultures were grown at 30°C while agitating continuously (200 rpm), after which culture filtrates were assayed for MuIFN-γ expression using enzyme-linked immunosorbent assay (ELISA).

Vector Construction

Total RNA was extracted from the mitogen-stimulated mouse splenocytes according to the procedure described previously [8]. RT-PCR was performed with primers pIFN-γF (5'-TGTTACTGCCACGGG-3') and pIFN-γR (5'-CGGTC-GACTCAGCAGCGACT-3') specific to the 5' and 3' ends of the cDNA sequence for mature MuIFN-γ (GenBank accession number K00083), respectively. The PCR amplicon was cloned into plasmid pGEM-T according to the manufacturer's recommendations (Promega, Madison, WI, U.S.A.) and the sequence encoding the signal peptide of amylase 1A (*Ramy1A*) (GenBank accession number X16509) from rice [16, 19] was fused to the cDNA for the mature *MuIFN-γ* gene using an overlap extension method [10]. The fused construct was then cloned into pGEM-T vector, and the resulting plasmid was digested with *Bam*HI and *Sal*I. The digested 510-bp fragment containing the MuIFN-γ gene sequence was placed between either one of two promoters and the galactose-1-phosphate uridyl transferase (*GAL7*) terminator (GenBank accession number M12348) of a yeast episomal shuttle vector, YEp352 [21]. The *GPD* (glyceraldehyde-3-phosphate dehydrogenase) promoter (GenBank accession number M13807) and a yeast hybrid (AG) promoter consisting of the upstream activating sequence (UAS) of *ADH2* (alcohol dehydrogenase 2) (GenBank accession number J01314) and the *GPD* TATA element were described previously [21]. The resulting plasmids were denoted pYEGMuIFN-γ and pYEAGMuIFN-γ, respectively (Fig. 1). *S. cerevisiae* 2805 was then transformed with the expression plasmids according to the lithium acetate procedure [12]. The stability of the introduced plasmids in yeast was measured as previously described [19].

Expression and Quantitative Assay of MuIFN-γ

Expression of an introduced *MuIFN-γ* gene in transformed cells was confirmed by Northern blot analysis as previously described [21]. The probe was labeled with α-[^{32}P]-dCTP using a random labeling kit (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) [9], and hybridization was conducted in Church buffer [7% (w/v) SDS, 1% BSA, 1 mM EDTA, 250 mM $NaPO_4$, pH 7.2] at 65°C [4].

The concentration of a protein product of *MuIFN-γ* gene in the culture filtrate of *S. cerevisiae* was measured by sandwich ELISA. In this experiment, a baculovirus-infected insect cell-derived MuIFN-γ [(rMuIFN-γ) purchased from BD PharMingen (Franklin Lakes, NJ, U.S.A.)] was reconstituted in pure water to a concentration of 0.5 mg/ml according to the manufacturer's instructions and used as a standard. The culture filtrates of transformed yeasts were subsequently

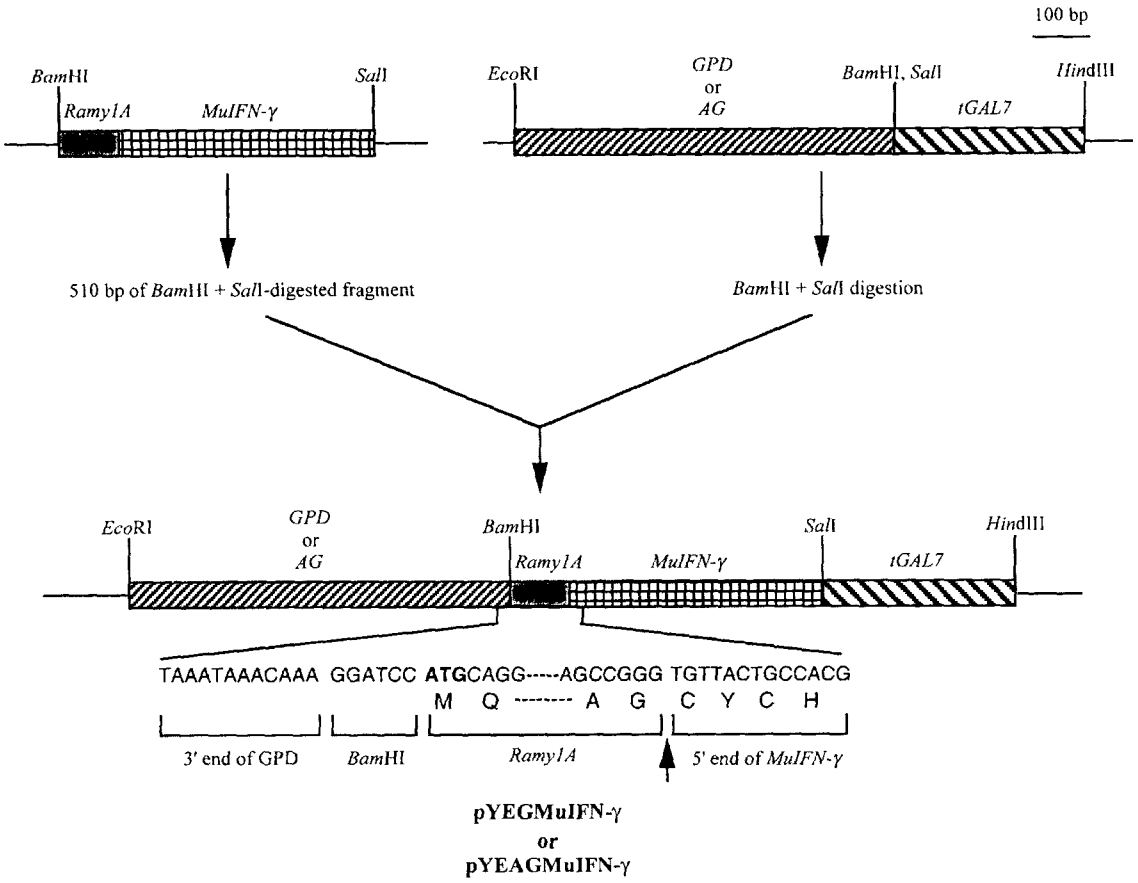

Fig. 1. Schematic diagram of the construction of transforming plasmids.
The boxes represent genes or their corresponding functional domains as described in the text. The sequence covering the links of promoter-signal peptide-MuIFN-γ is presented and the translation start codon is marked in bold letters. The arrow indicates the natural cleavage site of signal peptide of amylase 1A.

tested in an ELISA according to the manufacturer's instructions (BD PharMingen).

Western blot analysis was performed according to the procedure described previously [21]. The anti-MuIFN-γ antiserum was purchased from BD PharMingen and an anti-mouse IgG conjugated to a horseradish peroxidase was used as a secondary antibody. 4-Chloro-1-naphthol was applied as a substrate for colorimetric detection by horseradish peroxidase according to Sambrook *et al.* [24].

Measurement of Biological Activity of MuIFN-γ

Culture filtrates of recombinant *S. cerevisiae* were collected, dialyzed twice against PBS for 2 h at 4°C, and sterilized by passing it through a 0.2-μm syringe filter. The protein concentration in filter-sterilized culture filtrates was measured by the Bradford assay using the Bio-Rad Protein Assay Kit [2] and adjusted to 20 μg/ml for further analyses. An aliquot of 100 μl of each sterile culture filtrate was serially diluted with PBS and used for the bioassays. In order to measure the biological activity, 100 ml of mouse L929 (ATCC CCL-1) cell suspension (3×10^6 cells/ml) was mixed in the well of a 96-well microtiter plate (Falcon, Franklin Lakes, NJ, U.S.A.) with 100 ml of either MuIFN-γ standard (BD PharMingen Inc.) or our samples. The microtiter plate was incubated for 24 h at 37°C in a 5% (v/v) CO_2/air humidified atmosphere. After the inoculation of the cell with encephalomycarditis virus (EMCV), we measured the protected-host cell by MuIFN-γ using an amido blue-black staining method [6].

RESULTS AND DISCUSSION

Analysis of Transformed *S. cerevisiae*

Ten to twenty transformants of *S. cerevisiae* harboring each recombinant plasmid were selected on ura$^-$ medium. The plasmid DNAs from these transformations were isolated and reintroduced into *E. coli* to confirm the presence of recombinant plasmid in yeast. Northern blot analysis of 3-day cultured transformants with either pYEAGMuIFN-γ or pYEGMuIFN-γ revealed that all transformants showed the transcript of introduced *MuIFN-γ* gene (data not shown). Northern blot analyses indicated that trasnformants with pYEAGMuIFN-γ showed higher expression of *MuIFN-γ*

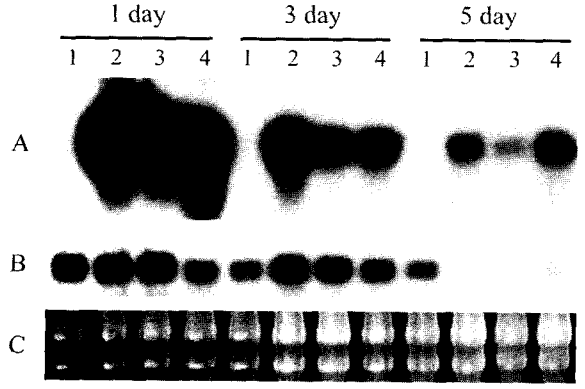

Fig. 2. Expression pattern of *MuIFN-γ* in TYEAGMuIFN-γ-1, -2, and -3.
Northern blot analysis using *MuIFN-γ* (A) and *GPD* (B) as probes were conducted. Lanes 1, 2, 3, and 4 contain the total RNA of recipient, TYEAGMuIFN-γ-1, -2, and 3 strains, respectively. Culture periods are indicated at the top of the lanes. The ethidium bromide-stained gel (C) indicates that a similar amount of RNA has been loaded for each sample.

gene than those with pYEGMuIFN-γ (data not shown), which were in good agreement with the following ELISA results. In addition, there was a variation at the transcription level of *MuIFN-γ* gene among transformants with the identical episomal expression vector, which was not unusual, when episomal 2μ ori-based plasmids are used [20, 23]. Therefore, transformants TAGMuIFN-γ-1, -2, and -3 harboring pYEAGMuIFN-γ were selected because of their high expression levels and examined for further experiments.

The expression of the *MuIFN-γ* gene in an expression medium YEP-EtOH was examined by Northern blot analysis (Fig. 2A). The accumulation of *MuIFN-γ* transcript peaked at 1-day cultures of three tranformants, but a rapid decline of the amount of *MuIFN-γ* transcript was observed thereafter, which appeared to be characteristic of the expression pattern of the yeast hybrid (AG) promoter [18]. The expression pattern of the *GPD* gene in the YEP-EtOH medium was examined as an internal control (Fig. 2B). The *GPD* expression differed from that of *MuIFN-γ*: it remained at a maximum until the culture was three days old and then gradually decreased. Since there was a rapid decline in the target gene expression, the growth curve in YEP-EtOH medium was measured for each of the transformed yeasts and compared with that in YEPD medium (Fig. 3). The growth patterns of all three transformants were similar to that of the host strain, indicating that no growth abnormalities of transformant due to the expression of MuIFN-γ were observed. In addition, the growth curves in both YEPD and YEP-EtOH media showed a canonical sigmoid curve: after the first three days of exponential growth, both entered the stationary phase at 4-day culture and then gradually decreased. However, the growth in YEP-EtOH medium was lower than that in YEPD medium and a 20% to 30% reduction was observed in the YEP-EtOH medium.

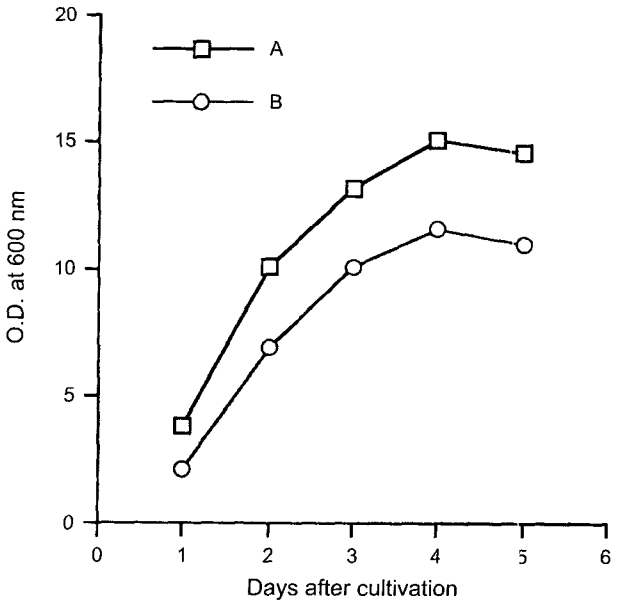

Fig. 3. Time courses of cell growth in YEPD (A) and YEP-EtOH (B) media.
No differences among strains of recipient and transformants were observed. Data are shown as a representative profile based on duplicated experiments with three replications.

Production of MuIFN-γ

The production of MuIFN-γ protein was examined using ELISA. Unlike the transcription of *MuIFN-γ* gene, the

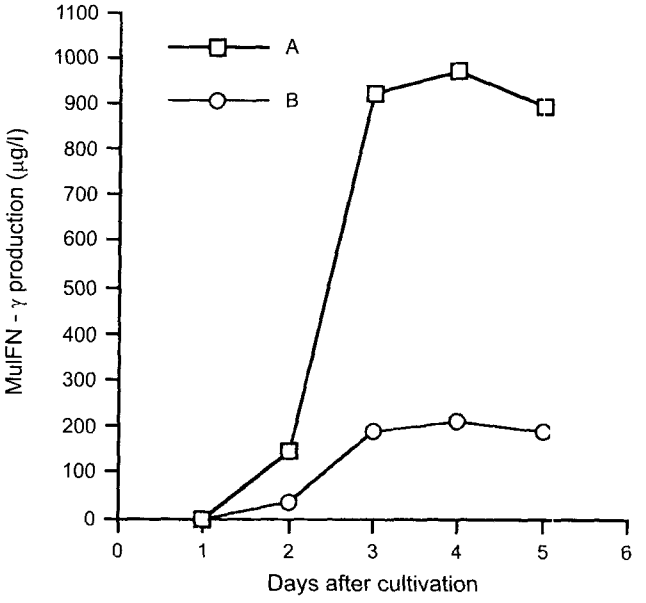

Fig. 4. Comparison of MuIFN-γ production using recombinant *MuIFN-γ* fused with different promoters.
(A), MuIFN-γ production with *AG* hybrid promoter (TYEAGMuIFN-γ); (B), MuIFN-γ production with *GPD* promoter (TYEGMuIFN-γ) Data are shown as a representative profile based on duplicated experiments with three replications.

maximum yield of MuIFN-γ was found in the stationary phase, which occurred at 4 days after cultivation; yields were 1.0 mg and 0.2 mg of MuIFN-γ per liter of expression media for the AG and *GPD* promoters, respectively (Fig. 4). The strength of promoters in this study differed slightly from the previous one [18] in that the GPD promoter gave the highest expression level of the heterologous gene. However, previous studies on glucose oxidase expression showed similar results with regard to promoter strength [21]. Thus, the difference in the promoter strength could be due to the gene of interest rather than the intrinsic differences in the promoter strength.

When cells were grown in YEPD containing 1.5% ethanol (YEPD-EtOH), five-times lesser amount of the recombinant MuIFN-γ (0.2 mg/l of MuIFN-γ) was observed, compared with that grown in YEP-EtOH (Figs. 5A and 5B). However, the YEPD-EtOH supported better growth of recombinant yeast and 30% more cell density was obtained, as compared with YEP-EtOH. Therefore, it appeared that a proper induction of gene expression under the inducible hybrid promoter was not achieved, using YEPD-EtOH instead of YEP-EtOH, and the residual amount of sugar (≤2%) was enough to repress the AG promoter even in the presence of ethanol.

Since it has been established that the production of the target protein was favorable if cells were grown at the nonselective condition prior to the inoculation into a production medium, the productivity of the primary cultures from YEPD and uracil selective medium was compared. As shown in Fig. 5C, five times greater amount of the recombinant MuIFN-γ (5.0 mg/l of MuIFN-γ) was obtained, using the cells from YEPD rather than the uracil selective medium (Fig. 5B). The plasmid stability was measured by plating the cells on YEPD and uracil selective medium. When YEPD was used to obtain cells as an inoculum for the production medium, the plasmid-harboring cell was around 50% after 5-day incubation in the production medium, which was lower than in the previous studies (≥85%) using uracil selective medium for the production of primary culture. Therefore, an inoculation to the production medium was performed as follows; transformed cells were enriched by growing a single colony in a test tube containing 5 ml of uracil selective medium for 24 h, 250 μl of cell suspension was transferred into a tube containing 5 ml of YEPD followed by 12 h incubation, and 2×10^8 cells were inoculated into a production medium (YEP-EtOH). Using these inoculation processes, the transformed cells were more than 95% at the end of 5 ml culture of YEPD and 75% of the cells retained plasmid after 5-day incubation in a production medium. Moreover, the productivity of MuIFN-γ increased to 6.5 mg/l of culture medium (Fig. 5D), which was the highest yield in all the trials. These results indicated that the less-stressed cells gave a better yield of target protein and it will be of importance to maintain a high proportion of transformants in the expression stage.

Considering the high level of transcript at the beginning of the production culture and the high protein level after a 3-day culture, it appeared that MuIFN-γ was secreted and preserved as stable in a culture medium due to the absence of major protease in the host strain.

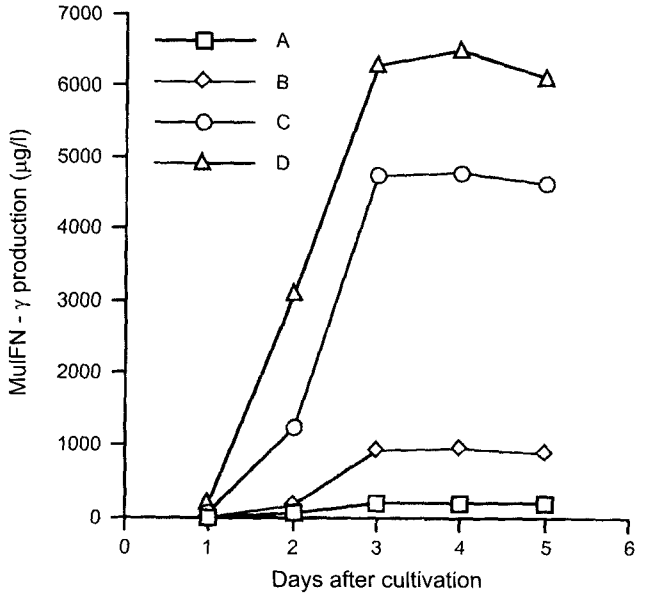

Fig. 5. Comparison of MuIFN-γ production depending on culture conditions.

(A), MuIFN-γ production in YEPD medium using an inoculum prepared from a culture in ura⁻ medium; (B), MuIFN-γ production in YEP-EtOH medium using an inoculum prepared from a culture in ura⁻ medium; (C), MuIFN-γ production in YEP-EtOH medium using an inoculum prepared from a culture in YEPD medium; (D), MuIFN-γ production in YEP-EtOH medium using an inoculum prepared from successive cultures in ura⁻ and YEPD media (refer to text). Data are shown as a representative profile based on duplicated experiments with three replications.

Measurement of Biological Activity of MuIFN-γ

The biological activity of MuIFN-γ protein from culture filtrates of recombinant TYEAGMuIFN-γ was analyzed by its ability to reduce the viral killing of target cell types by inhibiting the replication of an infecting virus. Neither of the negative controls, which included the culture filtrate from a recipient strain and a mock transformant, showed any protection against virus infection. Additionally, PBS had no effect on viral inhibition (Fig. 6). In contrast, the positive control rMuIFN-γ, as well as the culture filtrates of TYEAGMuIFN-γ, exhibited antiviral activity.

No detectable biological activity was observed using a sample of cytosolic proteins of TYEAGMuIFN-γ (data not shown). These results indicated that MuIFN-γ is expressed and secreted as a biologically active form from TYEAGMuIFN-γ.

In our Western blot analysis, two MuIFN-γ bands reacted with anti-MuIFN-γ antibody and their masses were determined to be 21 kDa and 23 kDa (Fig. 7). Two forms

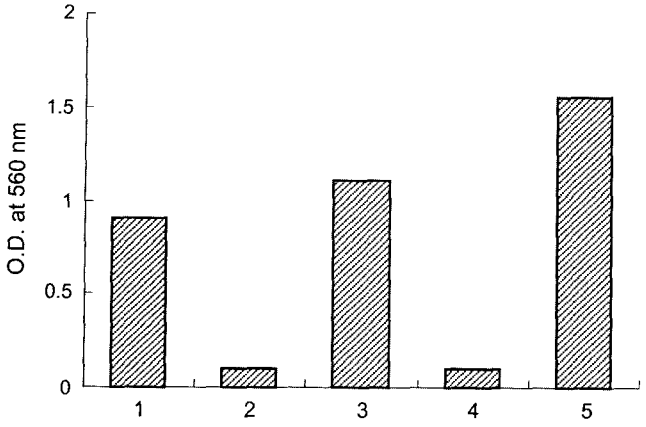

Fig. 6. Bioassay of yeast-derived MuIFN-γ.
Protected-cell growth is represented as O.D. at 560 nm. Bars 1, 2, and 3 indicate results with the protein preparations from yeast-derived MuIFN-γ, mock-transformant, and MuIFN-γ standard, respectively. Bars 4 and 5 represent the cell growth treated with EMCV only and media as negative and positive controls, respectively. Data are shown as a representative profile, based on duplicated experiments with three replications.

of polypeptides containing antiviral activity were also observed in human MuIFN-γ and these were due to differences in their degree of glycosylation. Since the encoded size of the mature MuIFN-γ based on the DNA sequence is only 15,894 Da and the purchased MuIFN-γ showing equal or faster mobility than the yeast-expressed MuIFN-γ is known to be glycosylated, the MuIFN-γ in this study appears to be glycosylated and there are at least two forms of MuIFN-γ with different degrees of glycosylation as well. Sequence analysis of the *MuIFN-γ* gene revealed two potential N-glycosylation sites at 19 and 72 residues of a matured protein [11]. Further studies, however, need to be carried out to determine the sites and types of glycosylation which occurred in the MuIFN-γ.

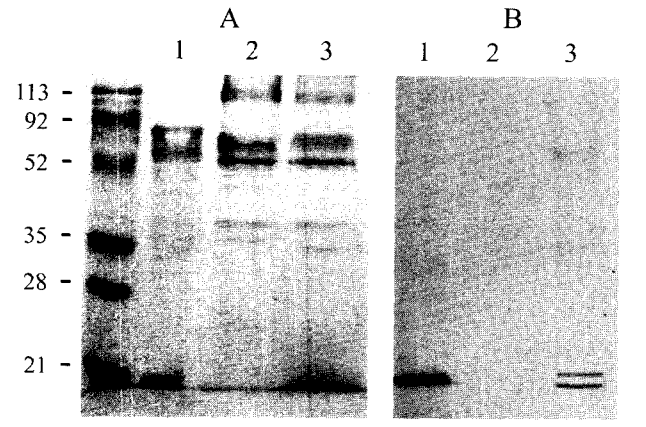

Fig. 7. Western blot analysis of a recombinant MuIFN-γ.
A, Coomassie blue stained SDS-PAGE of culture filtrate of yeast. B, Antigen-antibody reaction of the corresponding twin gel of culture filtrate of yeast. Lane 1, 10 μl (0.2 μg/μl) of standard MuIFN-γ from BD PharMingen; lane 2, 10 μl (1.3 μg/μl) of concentrated culture filtrate of host cell; lane 3, 10 μl of concentrated culture filtrate of transformant TYEAGMuIFN-γ-1. Numbers on left refer to the estimated sizes in kDa.

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