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33. An antibody to rabies glycoprotein was raised against the glycoprotein-containing NP-40 supernatant fraction from purified rabies virions. Rabbits were injected once and then again at 3 weeks; serum titers (all 1:30,000, 3 weeks after the second injection) were determined by immunofluorescent neutralizing antibody assay [J. B. Thomas, in *The Natural History of Rabies*, G. M. Baer, Ed. (Academic Press, New York, 1975), p. 401]. A single crude serum sample from a single rabbit (titer 1:34,800) was diluted 1:300 to 1:1000 for use in ELISA or for filter hybridization. In some experiments, antiserum was first adsorbed to control bacterial extracts to eliminate nonspecific reaction in the ELISA.
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35. Sample antigens to be assayed were diluted into phosphate-buffered saline (PBS), and 100 μl was pipetted onto the wells of a Falcon flexible assay plate. After a 30-minute incubation period, unbound sample was aspirated away, and 100 μl of PBS plus 0.25 percent gelatin was applied. After 15 minutes, the gelatin solution was removed and the wells were washed three times with PBS plus 0.05 percent Tween. Rabbit antiserum to rabies glycoprotein (100 μl) diluted in PBS-Tween was added and incubated for 30 minutes. After washing three times in PBS-Tween, 100 μl of goat antiserum to rabbit immunoglobulin G–alkaline phosphatase conjugate was added (Zymed Laboratories; diluted 1:1000 in PBS-Tween just prior to use). After 30 to 60 minutes of incubation and washing, 100 μl of substrate (*p*-nitrophenyl phosphate, reconstituted according to Zymed instructions) was added. The absorbance at 405 nm was determined on a Dynatech ELISA reader. The standard plot for each experiment was authentic rabies glycoprotein diluted in duplicate from 5 to 50 ng per well.
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42. All antibody binding steps and washings were done in 50 m*M* tris, *p*H 7.4, 0.15*M* NaCl, 5 m*M* EDTA, 0.25 percent gelatin, and 0.05 percent NP-40 at room temperature for 1 to 2 hours.
43. We thank George Baer and Brian Holloway for developing the antiserum to glycoprotein, Larry Bock for carrying out the protein blot experiment (*41*), Avima Yaffe and Eric Patzer for help in setting up the ELISA, and Jeanne Arch and Alane Gray for help in manuscript preparation. We thank Art Levinson and Ron Wetzel for many helpful discussions. Supported by Genentech, Inc.

# Secretion of Human Interferons by Yeast

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William J. Kohr, Howard L. Levine, David V. Goeddel

*Summary.* Plasmids were constructed to direct synthesis of the human interferons IFN-α1, IFN-α2, and IFN-γ in the yeast *Saccharomyces cerevisiae.* Expression of IFN genes containing coding sequences for secretion signals resulted in the secretion of IFN activity. A large proportion of the IFN-α1 and IFN-α2 isolated from the yeast cell growth media had the same amino termini as the natural mature interferons, suggesting a removal of the signal sequences identical to that of human cells. These results show that a lower eukaryote, such as yeast, can utilize and process a human signal sequence.

With the advent of recombinant DNA technology, it has been possible to examine the expression and secretion of eukaryotic gene products by the prokaryote *Escherichia coli*. Talmadge *et al.* (*1*) have shown that hybrid gene products containing bacterial–eukaryotic secretion signals (β-lactamase–rat preproinsulin) can be processed to proinsulin, which is then found in the periplasmic space of *E. coli*. They have also shown that for these hybrid proteins the eukaryotic signal (amino-terminal protein sequence) alone is sufficient to obtain secretion and processing in *E. coli* (*2*). Using another *E. coli* expression plasmid, Fraser and Bruce (*3*) have observed expression and secretion of chicken ovalbumin into the periplasmic space of *E. coli*. However, the secretion signal for this protein is thought to be within the protein sequence and there is no cleavage during the process (*4*). These results suggest that secretion signals may be functionally similar for many organisms. In this article we examine the secretion and processing of mammalian interferon (IFN) gene products in the lower eukaryote yeast.

The study of protein secretion from *Saccharomyces cerevisiae* (yeast) has focused primarily on the acid phosphatase and invertase enzymes (*5*), which are secreted into the periplasmic space. These enzymes are expressed as precursors having hydrophobic amino-terminal signal sequences (*6*) that are subsequently removed during the secretion process. Schekman and Novick (*5*) have used these enzymes to examine the secretion pathway in temperature-sensitive secretion mutants (*sec*). Characterization of these mutants has led to the recognition of a pathway in yeast that is organelle-dependent (*5*) and similar to that observed for the mammalian exocrine cells (*7*). The yeast pathway appears to start with translation of the pre-protein at the endoplasmic reticulum where the presequence (amino-terminal signal peptide) is removed after passage through the membrane, and then transported to the Golgi where vesicle intermediates are formed. These vesicles then move to the bud of the plasma membrane where fusion occurs with subsequent release of protein from the cell.

Plasmids that allow expression of heterologous (non-yeast) genes in the lower eukaryote yeast have recently been developed. Hitzeman *et al.* (*8*), using the promoter of the highly expressed yeast alcohol dehydrogenase I gene (*9*) to initiate transcription, demonstrated the synthesis of human IFN-α1 in yeast. However, the IFN-α1 used in these experiments was modified so that the $NH_2$-terminal signal peptide coding sequence was replaced by an ATG translational start codon. Therefore the polypeptide produced was mature IFN-α1 (the form found after secretion from human cells); it was not secreted from the yeast cell.

We have recently constructed a different portable yeast promoter (*10*) from another highly expressed yeast glycolytic gene encoding 3-phosphoglycerate kinase (PGK). This gene was isolated, characterized, and sequenced (*11*). When the PGK promoter fragment was

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used in a yeast plasmid, the heterologous complementary DNA (cDNA) genes for both mature (secretion signal absent) and pre-IFN's (secretion signal present) have been expressed in yeast. The pre-IFN genes contain coding sequences for mature IFN's as well as extra amino-terminal protein sequences. These terminal sequences signal the secretion of the proteins from the human cell and are cleaved during the process to give mature IFN's (*12*). We describe below the expression, processing, and secretion of these pre-IFN gene products by yeast, using the PGK expression system.

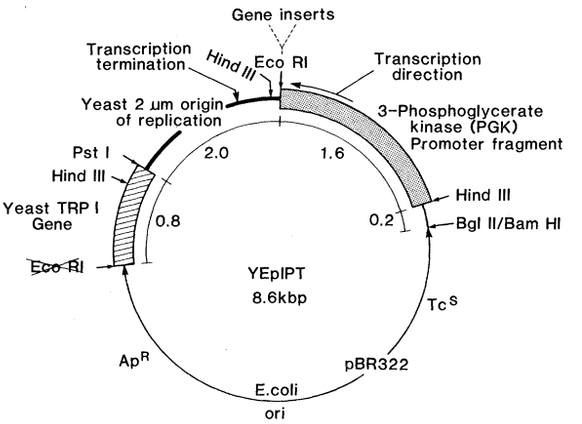


Fig. 1. Yeast expression plasmid. The partial restriction map of YEp1PT is shown. The components required for transcription and translation of a heterologous gene inserted at the single Eco RI site are designated.

### Heterologous Gene Expression Plasmid

All the IFN expression plasmids in our study were derived from a single parental plasmid. The plasmid YEp1PT, which contains the PGK promoter fragment and other expression, selection, and maintenance components (Fig. 1), including the large Eco RI to Bam HI fragment of pBR322 (*13*) containing the ampicillin resistance ($Ap^R$) gene and replication origin for selection and stable growth in *E. coli*. Also YEp1PT contains the *TRP*1 gene on an Eco RI to Pst 1 DNA fragment originating from yeast chromosome IV (*14*). This gene permits selection for the plasmid in *trp*1 mutant yeast cells growing in medium lacking tryptophan. The yeast origin of replication in YEp1PT is contained on a 2.0-kilobase-pair (kbp) fragment (Eco RI to Pst 1) from the endogenous yeast 2 micrometer (circumference) plasmid (*15*). This origin allows the DNA to replicate autonomously in yeast and be maintained as a plasmid in 90 to 95 percent of yeast cells grown in media depleted of tryptophan (legend to Table 1).

The YEp1PT plasmid system contains the yeast PGK promoter fragment (Hind III to Eco RI). Transcription originates from the PGK promoter near the only Eco RI restriction site in the plasmid [the other Eco RI site was removed (*8*)] and proceeds in the direction indicated (Fig. 1). The PGK promoter fragment was constructed from the yeast PGK gene based on DNA sequence information (*11*) and contains about 1600 base pairs (bp) of PGK 5′-flanking DNA sequence. The sequence 5′-TCTAGAATTC-3′ (T, thymine; C, cytosine; G, guanine; A, adenine) which contains Eco RI and Xba I restriction sites for convenient attachment of heterologous genes (*12*), has been substituted for the 10 bp of PGK sequence preceding the ATG translation initiation codon. A Hind III–Bgl II fragment from the yeast *TRP*1 gene region (*14*) was used as a Hind III to Bgl II converter for ligation with the Bam HI site of pBR322. The 2.0-kbp fragment from 2 μm plasmid DNA also contains the transcription termination and polyadenylation signals which are normally the signals for the "Able" (or *FLP*) gene in the 2 μm plasmid (*16*). We have recently shown that such a region is essential for heterologous gene expression in yeast (*17*), which Zaret and Sherman (*18*) have shown for homologous gene expression.

Each of the IFN genes was inserted as an Eco RI restriction fragment into the only Eco RI site of YEp1PT between the promoter and termination signals. Restriction mapping allowed us to select isolates in which the gene was oriented correctly for transcription from the PGK promoter. Other foreign genes, which have been expressed in yeast when this system was used include the genes for human serum albumin, hepatitis B virus surface antigen, and human growth hormone (*10*).

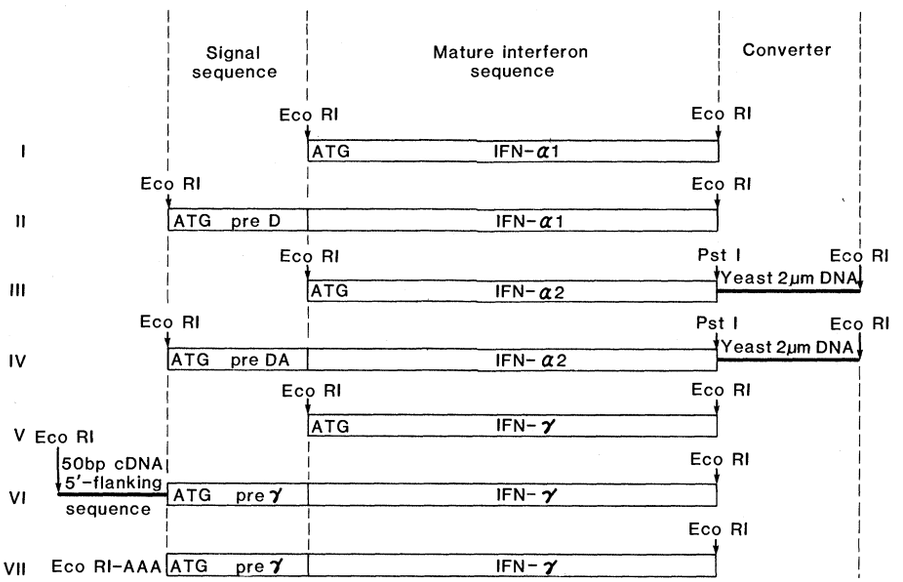


Fig. 2. Modifications of IFN cDNA's for insertion into the Eco RI site of YEp1PT. Boxed regions refer to IFN cDNA sequences and are not drawn to scale. Line sequences show unique characteristics or additional DNA added to the cDNA's. The addition of Eco RI restriction sites and ATG translational starts immediately before the mature IFN sequences have been described for constructions I (*8*), III (*19*), and V (*20*). The Eco RI site in construction II was added by cleavage of the IFN-α1 signal peptide coding region (*12*) with Hae III and subsequent ligation to the synthetic deoxyoligonucleotide 5′ CCATGAATTCATGG 3′, which regenerates the rest of the signal sequence and an Eco RI site as in the mature IFN constructions. Hybrid construction IV was made with the use of a Dde I site common to the signal coding regions for preD IFN-α1 and preA IFN-α2 (*12*) (see Fig. 3). Construction VI was made by conversion of Sau 3A sites in the 3′ and 5′ flanking regions of IFN-γ cDNA (*20*) to Eco RI sites by means of converter fragments in plasmid pUC7 (*29*). The 5′ Eco RI site of construction VII was made with synthetic DNA in a primer repair reaction (*22*). Restriction enzyme cleavage, synthetic DNA synthesis, and ligations were done as described (*11*). The *E. coli* K-12 strain 294 (*30*) was used for all bacterial transformations. Purification of covalently closed circular plasmid DNA's from *E. coli*, transformation of *E. coli*, and plasmid miniscreens were done as described (*11*).

### Modifications of IFN Complementary DNA's for Expression in Yeast

Some of the Eco RI restriction fragments, containing interferon cDNA's which were constructed for use in the YEp1PT expression plasmid, are illustrated in Fig. 2. The constructions of the DNA fragments for mature IFN-α1 (*8*), IFN-α2 (*19*), and IFN-γ (*20*) are known (*21*). The mature IFN-α2 construction required a final modification to convert the Pst I restriction site at the 3′ end of the gene to an Eco RI site. A 245-bp Pst I to Eco RI DNA fragment obtained from yeast 2 μm plasmid DNA (*16*) was used for this purpose. Eco RI sites were placed immediately upstream from the ATG initiation codons of the pre-IFN genes by the primer repair technique (*22*) or by conversion of convenient restriction sites. The amino-terminal protein sequence of each IFN (*21*) and the cleavage site recognized during secretion from human cells is shown in Fig. 3. The gene for preD IFN-α1 (construction II) was modified for direct expression by the primer repair method. The preD/A IFN-α2 fragment (construction IV) was then made with a Dde I restriction site common to the signal peptide coding sequences of both the preD IFN-α1 and preA IFN-α2 genes. Amino acids that differ in preA from preD signal sequences are underlined in Fig. 3. The hybrid signal sequence (preD/A) of preD/A IFN-α2 is more like preD than preA, differing only at the −2 residue from preD. Two different preγ IFN-γ cDNA fragments were constructed. The Sau 3a restriction sites on both sides of the structural gene were converted to

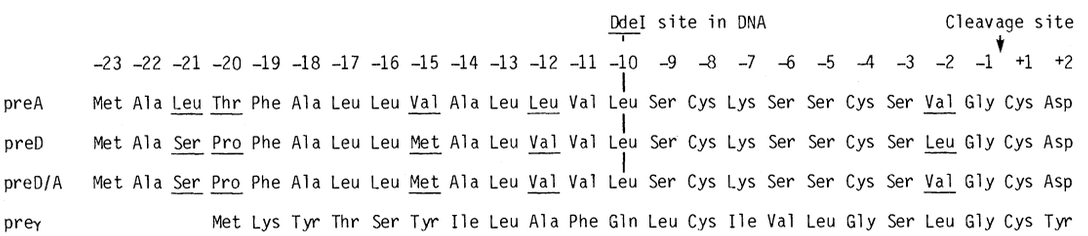


Fig. 3. Amino acid sequences of the various secretion signals. A comparison of the amino acid sequences (*28*) of the signal regions preD, preA, preD/A, and preγ is shown. The amino acids underlined represent differences between the amino acid sequences of preD and preA. The Dde I site indicates the junction shared by these two presequences used to prepare the hybrid (preD/A) presequence. The sites of cleavage used in human cells are also shown.

Table 1. Interferon expression. Yeast strains 20B-12 (α *trp1 pep4-3*) (*24*) and GM3C-2 (*a leu2-3 leu2-112 trp1-1 his4-519 cyc1-1 cyp3-1*) (*23*) were used for yeast transformations (*33*). Yeast were grown on the following media: (i) YEPD contained 1 percent yeast extract, 2 percent peptone, and 2 percent glucose (used prior to transformation). (ii) YNB+CAA (used for $Trp^+$ selection and plasmid maintenance) contained (per liter) 6.7 g of yeast nitrogen base (without amino acids) (YNB) (Difco), 10 mg of adenine, 10 mg of uracil, 5 g of Difco Casamino acids (CAA), and 20 g of glucose. Solid medium contained 3 percent agar. Stability of plasmids in yeast was determined by replica plating colonies from YEPD (nonselective) to YNB+CAA (selective) media. Extracts and media from yeast were assayed for interferon by the cytopathic effect (CPE) inhibition assay and comparison with interferon standards (*25*). Media were assayed directly after cell removal while yeast extracts were prepared as follows: cultures were grown in YNB+CAA and 10-ml samples of cells were collected by centrifugation, resuspended in 3 ml of 1.2*M* sorbitol, 10 m*M* $KH_2PO_4$ (*p*H 6.8), and 1 percent zymolyase 60,000, and incubated at 30°C for 30 minutes (about 90 percent of cell wall removed). Spheroplasts were centrifuged, resuspended in 150 μl of 7*M* guanidine hydrochloride, and diluted in phosphate-buffered saline (PBS) containing bovine serum albumin (BSA) [20 m*M* $NaH_2PO_4$ (*p*H 7.4), 150 m*M* NaCl, and 0.5 percent BSA]. Alternatively, 10 ml of cells at the same $A_{660}$ were centrifuged and resuspended in 0.4 ml of 7*M* guanidine hydrochloride in an Eppendorf (1.5 ml) tube containing about 0.4 ml of glass beads (0.45 to 0.5 mm; B. Braun Melsurgen AG). These tubes were mixed twice for 2 minutes at the highest Vortex setting; they were cooled in ice between the mixings. The extracts were cleared in an Eppendorf centrifuge (0.5 minute) and diluted in PBS/BSA buffer as above. Bioassays were performed with MDBK cells (*25*) for IFN-α1, IFN-α2, and their respective pre-forms, but with HeLa cells (*25*) for IFN-γ and preγ IFN-γ.

| Gene construction | Eco RI fragments | Yeast | Inside cell* | | Released after cell wall removal† | | Outside cell (media) | | Final‡ $A_{660}$ | Activity secreted§ (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | $A_{660}$ = 1 ($10^6$ U/liter) | Total cell protein (%) | $A_{660}$ = 1 ($10^6$ U/liter) | Total cell protein (%) | $A_{660}$ = 1 ($10^6$ U/liter) | Total cell protein (%) | | |
| I | IFN-α1 | GM3C-2 | 130 | 1.0 | 0 | 0 | 0 | 0 | 1.0 | 0 |
| II | preD IFN-α1 | GM3C-2 | 27 | 0.3 | 0.4 | 0.004 | 0.8 | 0.008 | 1.4 | 4 |
| III | IFN-α2 | 20B-12 | 130 | 1.0 | 0 | 0 | 0 | 0 | 1.0 | 0 |
| IV | preD/A IFN-α2 | 20B-12 | 19 | 0.2 | 0.5 | 0.005 | 0.5 | 0.005 | 1.0 | 5 |
| IV | preD/A IFN-α2 | 20B-12 | 25 | 0.1 | | | 2.0 | 0.007 | 3 to 4 | 8 |
| IV | preD/A IFN-α2 | GM3C-2 | 28 | 0.3 | 0.3 | 0.003 | 0.5 | 0.005 | 1.3 | 3 |
| V | IFN-γ | 20B-12 | 0.6 | | | | 0 | 0 | 1.0 | 0 |
| VI | preγ IFN-γ + cDNA 5′ flanking sequence | 20B-12 | 0.2 | | | | 0.03 | | 1.2 | 15 |
| VI | preγ IFN-γ + cDNA 5′ flanking sequence | GM3C-2 | 0.38 | | | | 0.06 | | 0.93 | 16 |
| VII | preγ IFN-γ | 20B-12 | 0.9 | | | | 0.19 | | 1.0 | 21 |
| VII | preγ IFN-γ | GM3C-2 | 1.9 | | | | 0.19 | | 0.93 | 10 |

*Two methods were used for extracts. When cell walls were removed the "inside cell" amount was really inside material; however, when cell walls were not removed, the "inside cell" amount and the "released after cell wall removal" are both part of "inside cell" amount—this type of extract involves glass beading cells without cell wall removal. Glass bead extracts, without cell wall removal, were always made for IFN-γ and preγ IFN-γ and PBS buffer was used instead of 7*M guanidine hydrochloride*. The specific activities of IFN-α1 and IFN-α2 were both assumed to be $10^8$U per milligram of protein for the calculations. One liter of yeast culture contains about 100 mg of protein at an $A_{660}$ = 1. †See procedure for cell wall removal above. ‡Absorbance at 660 nm of culture at each assay done. §The activity secreted is the percent "released after cell wall removal" plus the percent "outside cell". When cell walls were not removed, the "activity secreted" does not include the periplasmic activity.

Eco RI sites in one construction. The resulting fragment (VI) contains 50 bp of 5′ untranslated DNA sequence. The other construction (VII) was made with a synthetic DNA converter. The signal sequences (Fig. 3) all have regions of hydrophobic residues which are characteristic of other signal sequences (*7*).

### Expression of Interferon Complementary DNA's in Yeast

The genes shown in Fig. 2 were inserted into YEp1PT at the Eco RI site, and the *trp*1⁻ yeast strains GM3C-2 (*23*) and 20B-12 (*24*) were transformed with these plasmids. Yeast strain 20B-12 was chosen for its diminished protease activity, which might result in more stable secreted IFN molecules. Transformants were assayed for interferon activity by the cytopathic effect test (*25*). The assays were done on three distinct compartmental locations in the yeast culture (Table 1).

Interferon activity remaining inside the cell after cell wall removal is defined as interferon activity that is not secreted. Interferon activity found in the medium (material completely separate from yeast cell) plus the activity released from the cells after cell wall removal by the enzyme zymolyase (*5*) together represent the total secreted material. Alternatively, when cell walls were not removed, "inside cell" activity also includes the secreted activity present in the periplasmic space; thus, under these conditions, the "activity secreted" may be an underestimate (Table 1).

Both mature IFN-α1 and IFN-α2 genes (constructions I and III) were expressed in the yeast as 1.0 percent of the total cellular protein based on IFN activity bioassays. This calculated level was confirmed by sodium dodecyl sulfate (SDS)–polyacrylamide gel protein analysis of whole cell extracts (data not shown). No activity was found when the genes were inserted in the wrong orientation. No secretion of IFN activity was observed with these two mature genes or with the mature IFN-γ gene (construction V). However, IFN activity was found in the media for all of the pre-IFN constructions.

As shown in Table 1, IFN secretion varies from one gene to another with preγ IFN-γ giving the highest percentage of secretion. Depending on yeast strain and the gene construction, secretion into the medium of IFN-γ varies from 10 to 21 percent. However, additional IFN may be in the periplasmic space (not determined). Comparison of the production of preγ IFN-γ directed by constructions VI and VII shows that the presence of 50 bp of 5′ untranslated cDNA sequence reduces but does not destroy expression of the gene. However, this result is not inconsistent with the Kozak hypothesis of translation initiation in eukaryotes (*26*) since there are no translational start codons in this intervening region. Yeast containing preD IFN-α1 and preD/A IFN-α2 constructions secrete from 3 to 5 percent of IFN activity into the periplasmic space and medium at an $A_{660} \simeq 1$; however, when preD/A IFN-α2/20B-12 was grown to $A_{660}$ of 3 to 4, secretion into the medium alone was 8 percent. The amount of IFN activity in the periplasmic space was not determined at this higher absorbancy. However, in most instances the IFN activity in the periplasmic space was approximately equal to that found free in the media.

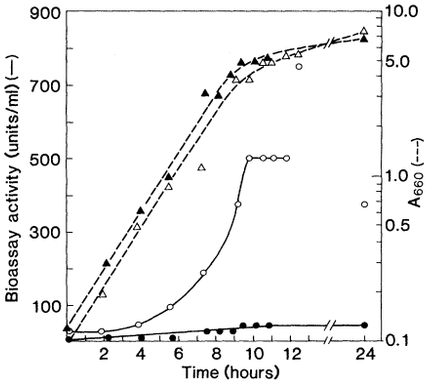


Fig. 4. Growth curves (triangles) showing secretion (circles) of preD/A IFN-α2 into the medium. YEpIPT-IFN-α2/20B-12 (▲, ●) synthesizes mature IFN-α2 which remains within the cell. YEpIPT-preD/A IFN-α2/20B-12 (△, ○) expresses the preD/A IFN-α2 gene and produces IFN activity in the medium. The media were assayed as described in the legend of Table 1.

The IFN's produced by two of the yeast strains were further characterized. These were YEp1PT-preD/A IFN-α2/20B-12 and YEp1PT-IFN-α2/20B-12. The former contains construction IV (Fig. 2) in the Eco RI site of YEp1PT and results in IFN-α2 activity inside the cell, in the periplasmic space, and outside the cell (medium). The latter yeast strain contains construction III (Fig. 2) in YEp1PT and produces mature IFN-α2 intracellularly but not in the medium. Growth curves for these two yeast strains in YNB plus Casamino acids (Difco) selective medium are shown in Fig. 4. Bioassays of the medium at various times during cell growth clearly demonstrate that the presequence on IFN-α2 is essential for secretion of IFN activity into the media. When the presequence is absent no activity is released. This has also been confirmed by further analysis of proteins from the two concentrated media. After separation of the proteins on an SDS–polyacrylamide gel and transfer to nitrocellulose paper (*27*), labeled antibody specific for interferon reacts with a protein the size of mature IFN-α2 only from the preD/A IFN-α2 expressor and not from the mature IFN-α2 expressor (data not shown). It is also evident that the activity in the medium and the percent secreted (Table 1) reach maxima near the stationary phase of cell growth; however, expression in the cell reaches a maximum during the log phase of growth (between $A_{660} = 1$ to 2, data not shown).

One possible explanation for finding IFN in the medium was that the presequence of interferon somehow makes the cells more susceptible to lysis during growth. This possibility was examined by measuring the amounts of protein in the media at stationary phase. The media from both yeast strains (containing constructions III and IV) contained equivalent concentrations of total protein when compared at the same cell density and showed identical patterns upon SDS-gel electrophoresis. Thus, true secretion of IFN must be occurring.

### Nature of Processing of Pre-IFN's

Since yeast cells can secrete preD/A IFN-α2 and since secretory processing of the amino-terminal end might occur as in mammalian cells, it was of interest to purify the protein product from the media and cells separately.

Cell extracts and media were obtained from 5-liter fermentations and the interferon was purified as described (Fig. 5). Cell extracts also contain the IFN from the periplasmic space since the cell wall was not removed prior to extract preparation. The protein sequencing results obtained for this purified preD/A IFN-α2 are shown in Fig. 5. The sequence expected for preD/A IFN-α2 if no processing were to occur and the normal cleavage point of this interferon in mammalian cells (*12*) are also shown. Two independent sequence runs were performed on two different purified samples from cells and media. Most of the IFN in the medium was properly processed (64 percent), but another form (36 percent) containing three additional amino acids of presequence was also present. The intracellular interferon also contained these two forms, but in slightly different proportions, as well as a third form containing eight amino acids of presequence. Full

length presequence was never observed, suggesting that yeast processes all of the pre-IFN in some manner.

It is possible that the processed form containing three amino acids of presequence resulted from the hybrid nature of the preD/A signal sequence. Therefore, the processing of the nonhybrid preD IFN-α1 protein was also examined. PreD is different from preD/A only at amino acid position −2 [Leu versus Val (*28*)]. When the processing of preD IFN-α1 purified from the medium was examined, both the +1 and −3 forms were again observed (Fig. 5). However, a minor species was also present in the medium as a −14 form, which was not seen for preD/A IFN-α2. It should be mentioned that preD IFN-α1 was produced from a high cell density yeast fermentation ($A_{550}$ = 60) unlike preD/A IFN-α2 ($A_{660}$ = 4). This difference in growth conditions may be responsible for the −14 form. Again, even at high cell density, there was no evidence of cell lysis occurring in the culture. Interestingly, at this high cell density, a higher percentage secretion (30 percent) into the medium was obtained for preD IFN-α1. High cell density fermentations of preD/A IFN-α2 expressing yeast also show this higher percentage secretion.

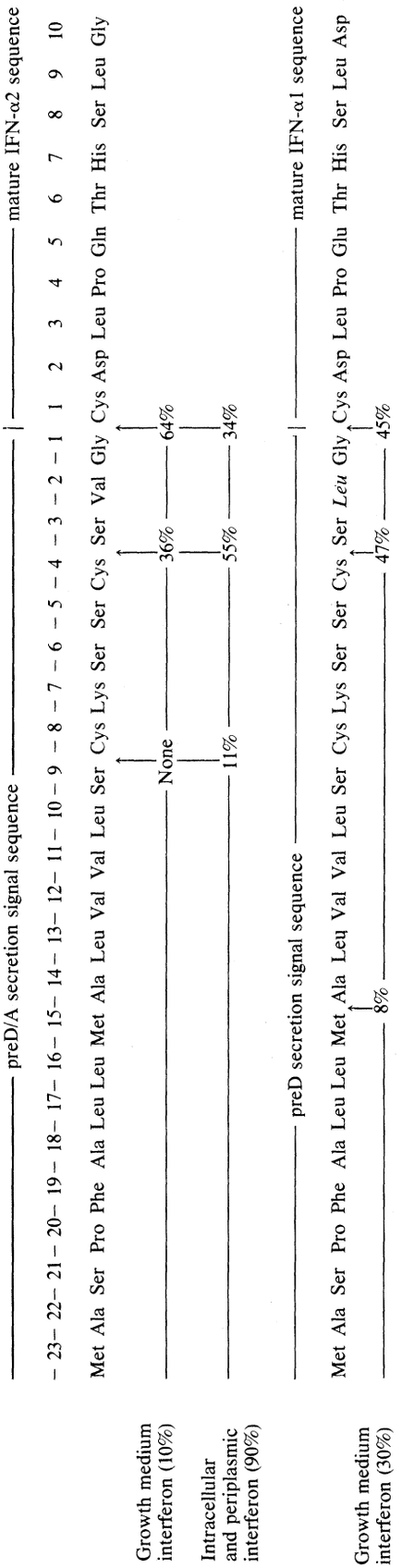


Fig. 5. Processing and distribution of preD/A IFN-α2 and preD IFN-α1 by yeast. The 23 amino acid presequence of preD/A IFN-α2 (consisting of 23 amino acids) is shown as well as the first ten amino acids of mature IFN-α2 sequence. Ten percent of the synthesized IFN activity was found in the medium and 90 percent was cell associated (about 80 percent in the cell and 10 percent in the periplasmic space). The IFN from these two growth fractions (medium and cells) was processed in various forms as shown. The preD IFN-α1 isolated from culture medium was processed as shown with 30 percent in the medium and 70 percent cell associated. Yeast were grown at 30°C to $A_{660}$ of 4. At this time the 5-liter culture was harvested by centrifugation. The medium was concentrated and diafiltered in an Amicon thin channel apparatus or a 2.5-liter stirred cell. The retentate was further purified by ion exchange chromatography on CM-52 and subsequent immunoaffinity column chromatography as described for IFN-α2 (*31*). Fractions containing IFN were further purified by high-performance liquid chromatography (HPLC) on a Synchropak RP-P column. The column was eluted at a flow rate of 1 milliliter per minute with a linear gradient of 0 to 100 percent acetonitrile containing 0.1 percent trifluoroacetic acid, *p*H 2.5, in 60 minutes. The protein in the peak fractions containing interferon activity was then sequenced at the amino-terminal end by Edman degradation (*32*). The cells were disrupted in a Bead Beater (Biospec Products). The lysate was centrifuged, the pellet was washed, and the supernatants were combined. The supernatants were dialyzed, and the IFN was purified as described above for medium material. The protein from fractions containing IFN activity was then sequenced at the amino-terminal end. The protein sequence was interpreted by noting which 3-phenyl-2-thiohydantoin (Pth) amino acid derivatives increased in each Edman cycle and then decreased in the following cycle (*32*). Pth amino acids that normally give low recoveries (Cys, Ser, Thr, Arg, His) were assumed during a cycle based upon amino acid sequence obtained in previous and following cycles. The percentage of each form was estimated by comparing the areas of the interpreted residue with an area from a standard mixture of Pth amino acids run on the same HPLC run. Most of the forms were sequenced for 21 cycles or 21 amino acids. An internal standard of norleucine was introduced in each chromatogram to ensure that retention times were reproducible.

## Conclusions and Discussion

To investigate the secretion of heterologous proteins from *S. cerevisiae*, we have constructed plasmids for in vivo transcription of the genes for several mature IFN's and several pre-IFN's. Whenever the coding sequences for hydrophobic signal peptides were present, IFN antiviral activity could be recovered both from the host cells and from the culture medium, while all of the IFN whose synthesis was directed by genes for mature IFN remained inside the cells. We have attempted to characterize the requirements for secretion by undertaking constructions in which the interferon gene would be provided with its own natural signal sequence, as in preD IFN-α1, or would be provided with a hybrid signal sequence designed as a composite of two IFN-α species, as in preD/A IFN-α2. While, in general, the yeast cells which harbored these constructions secreted IFN into the culture medium, the amount of activity differed between strains, and the IFN species purified and sequenced also differed.

Two forms of preD/A IFN-α2, together constituting 10 percent of the total IFN expressed, were purified from the medium of cells harboring the preD/A IFN-α2 gene. The major species was properly processed as in human cells (+1, Fig. 5), while another species had three additional amino acids or presequence (−3, Fig. 4). This second form may indicate a difference between yeast and human cell processing of secreted proteins. Alternatively, the −3 form might be a natural processed form of IFN previously unnoticed in preparations made from human leukocytes. The presence of this species appears not to be caused by the hybrid signal sequence, since interferon species examined from medium of cells harboring the preD IFN-α1 gene are also heterogeneous. In addition to the +1 and −3 forms, that medium contained a minor interferon species which included 14 amino acid residues of signal peptide (−14 in Fig. 5). The presence of this minor form is somewhat surprising because of the preD/A IFN-α2

results and its retention of part of the hydrophobic region of the presequence. The significance of this form requires further investigation.

Three forms of cell-associated IFN-α2, constituting 90 percent of the total interferon expressed, were purified from cells harboring the preD/A IFN-α2 gene. One form (34 percent) was properly processed (+1, Fig. 5), a second form (55 percent) contained three additional amino acids (−3, Fig. 5), and a third form (11 percent) contained eight additional amino acids (−8). The last form was not seen in media, while IFN with a full length presequence (*21*) was never observed in the cells or media. However, it is possible that this pre-protein could lack IFN activity and would be lost during the purification process. The approximately fivefold lower expression levels observed for leukocyte pre-IFN's compared to mature IFN's are suggestive of this possibility (Table 1). Alternatively, the lower expression levels observed may be the result of pre-protein instability.

Overall, yeast cells appear to process both the secreted and nonsecreted IFN. The amount of activity secreted varies depending on the growth stage of the cells, with maximum percentages occurring at stationary phase in shake flasks and at the end of high cell density fermentations (30 percent of IFN in medium). However, complete secretion of interferon has never been seen even though most of the intracellular IFN is processed as the extracellular IFN. It is possible that IFN may be entering various organelles instead of being released through the plasma membrane. To examine this, we are comparing cellular locations of interferon from both mature and pre-interferon–producing yeast.

Several features make yeast an attractive host microorganism for production of polypeptides. Yeast is capable of withstanding high hydrostatic pressure and does not lyse after death. At no time have we seen evidence of cell lysis in stationary phase cultures, or in cultures grown in fermenters to very high cell densities. Furthermore, the growth medium typically contains only five to eight major protein species of apparent size (probably glycosylated) greater than 50,000 daltons. This relatively "clean" medium (less than 0.5 percent of total yeast protein), combined with the resistance of yeast to lysis by external stresses, provides a good system for secretion studies. We have shown above that secretion of heterologous gene products by yeast can be effected by use of natural or hybrid human signal sequences.

### References and Notes


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28. Abbreviations for amino acid residues are Met, methionine; Ala, alanine; Leu, leucine; Ser, serine; Thr, threonine; Pro, proline; Phe, phenylalanine; Val, valine; Cys, cysteine; Lys, lysine; Gly, glycine; Asp, aspartic acid; Tyr, tyrosine; Ile, isoleucine; Gln, glutamine; Glu, glutamic acid; and His, histidine.
29. Plasmid pUC7 has an Eco RI site on both sides of a Bam HI site and was given to us by Dr. Joachim Messing of the University of Minnesota.
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