

Inducible high level synthesis of mature human fibroblast interferon in *Escherichia coli*

Erik Remaut, Patrick Stanssens and Walter Fiers

Laboratory of Molecular Biology, State University of Ghent, Ledeganckstraat 35, 9000 Ghent, Belgium

Received 7 June 1983; Accepted 27 June 1983

ABSTRACT

We have obtained high level synthesis in *Escherichia coli* of mature human fibroblast interferon using a plasmid vector that was designed to allow easy coupling of a DNA coding region to the initiator AUG of the replicase gene of the RNA phage MS2 cloned downstream of phage λ 's leftward promoter. The activity of the promoter can be regulated by temperature. Induced cells accumulated the interferon up to 4% of the total cellular protein. The biological activity of the product amounted to 4×10^9 international units per litre of culture. The synthesis of human fibroblast interferon was shown to drastically inhibit the growth rate of the bacterial host.

INTRODUCTION

In general, for a given DNA sequence to be expressed into a polypeptide, the sequence has to be recognized by the host as a transcriptional and translational unit. In *Escherichia coli*, the *lac* promoter (1,2,3), the *trp* promoter (4,5) and the leftward promoter P_L of phage λ (6,7,8) have been most frequently used to obtain efficient transcription of downstream gene sequences. Despite an overwhelming body of sequence data of known ribosome binding sites, our understanding of the specific factors that determine the efficiency of initiation at a given ribosome binding site is still very incomplete (for review see ref. 9). In the absence of clearly defined parameters that, in a predictable way, govern the rate of expression of a gene, we decided to adapt the initiating region of a known, well-expressed gene for easy insertion of any DNA sequence. Our earlier observation that the replicase gene of the RNA phage MS2, cloned downstream from the P_L promoter (10), was expressed to 35% of de novo protein synthesis as early as 30 min after induction prompted us to explore the MS2 replicase initiation region as a potential insertion site for foreign DNA sequences.

We describe the construction and use of a plasmid vector that allows easy insertion of any coding region downstream of the P_L promoter and the MS2 replicase initiator AUG in phase with the latter. As an example,

efficient synthesis in E. coli of human mature β -interferon is demonstrated.

MATERIALS AND METHODS

1. Bacterial strains and plasmids

The bacterial strains used in this study are listed in Table I. Unless otherwise mentioned, the bacteria were grown at 28°C in LB medium (1% Bacto tryptone; 0.5% Bacto yeast extract; 0.5% NaCl).

Plasmids pPLc28, pPLc24 and pPLc2819 were used as the source of DNA fragments and have been previously described (7,11).

2. DNA methodology

Restriction enzymes were obtained from New England Biolabs, Mass., or Boehringer, Mannheim, and used according to the manufacturer's specifications. T₄ DNA ligase was prepared from strain K12ΔH1Δtrp (pPLc2819) as described (11).

Treatment with S₁ nuclease (Sigma Chemical Company, St. Louis,) was performed at 10°C in 200mM NaCl, 1mM Zn-acetate, 50mM Na-acetate, pH 4.5, for 30 min with a ratio of 5 units of enzyme per μg DNA. Protruding 5'-ends were filled in with DNA polymerase I (Klenow fragment) (Boehringer, Mannheim) at 20°C in 10mM MgCl₂, 7mM β-mercaptoethanol, 10mM Tris-HCl at pH 7.4 in the presence of 100 μM of each of the four dNTP's.

Restriction fragments were eluted from agarose gels using the freeze-squeeze method (12).

Table I List of E. coli strains used

<u>Bacterial strain</u>	<u>Genetic characters</u>	<u>Source /reference</u>
K12ΔH1Δtrp	Sm ^R , lacZam, Δbio-uvrB, ΔtrpEA2(λNam7, Nam53, cI857, ΔH1)	ref 6
M5219	Sm ^R , lacZam, trpAam, (λbio252, cI857, ΔH1)	ref 7
K514(λ)	thr-1, leu-6, thi-1, supE44, lacY1, tonA21, hsr ⁻ , hsm ⁺	ref 25
MC1061	araD139, Δ(ara, leu)7697, ΔlacX74, galU ⁻ , galK ⁻ , hsr ⁻ , hsm ⁺ , strA	ref 26
SG4044	lac ⁻ , lonΔ100, Δ(gal-blu) strA	S. Gottesman

All strains are derivatives of E. coli K-12

3. Induction of the P_L promoter

The expression plasmids used in this study contain the leftward promoter (P_L) of phage λ . The plasmids were propagated in a strain (K12 Δ H1 Δ trp) that supplies a temperature-sensitive repressor, coded for by a resident defective prophage. The activity of the P_L promoter can be regulated by appropriate choice of the temperature of incubation; repression is virtually complete at 28°C and full induction is obtained at 42°C. The conditions for induction and detection of induced proteins have been described (7).

4. Detection of interferon activity in bacterial extracts

Induced cells were harvested by centrifugation and resuspended in 1% SDS, 1% β -mercaptoethanol; 5M urea; 30mM NaCl and 50mM Hepes-buffer pH 7.0 (13). The cells were lysed by immersion in a boiling water bath for 3 min. Cell debris was spun down at 12,000 g for 10 min. Serial dilutions of the lysate were made in Eagle's minimal essential medium containing 10% foetal calf serum. Interferon activity was measured using a protection assay of human FS4 cells against cytopathic effect (CPE) after challenge with EMC virus. CPE was recorded after 24 h. All titres were calibrated against the NIH human fibroblast interferon reference G023-902-527.

RESULTS

1. Construction of pPLc245

The initiator ATG codon of the MS2 replicase gene is followed by the sequence, TCGAA (14). The TaqI site can be converted into a SalI site if the TCGA sequence were followed by a C residue. To accomplish this a genuine SalI site was cleaved with TaqI and ligated to the TaqI site succeeding the ATG codon of the replicase. The details of the construction are outlined below.

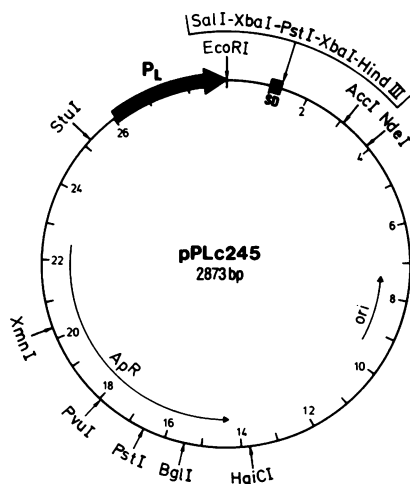
Plasmid pPLc2819 contains the multilinker sequence EcoRI - BamHI - HindIII - XbaI - PstI - XbaI - SalI - XbaI - HindIII (11). Plasmid pPLc2819 was cleaved with HaeII and the resulting fragments separated on a 1.4% agarose gel. A 600 bp fragment containing the multilinker sequence was recovered from the gel. After cleavage with HindIII and TaqI, two fragments with a HindIII on one end and a TaqI site on the other end were generated.

Plasmid pPLc24 contains the ribosome binding site and the N-terminal region of the MS2 replicase gene (7). pPLc24 DNA was digested with Bam HI and HincII. A 550 bp fragment encompassing the MS2 replicase ribosome binding site was eluted from an agarose gel and further digested with EcoRI and TaqI. The EcoRI site is located about 130 bp upstream from the ribosome binding site of the replicase. The TaqI sites are located immediately after the initiator ATG and at nucleotide 61 within the

replicase coding region.

Plasmid pPLc28 was cleaved with EcoRI and HindIII and served as the acceptor for an EcoRI - TaqI fragment and a TaqI - HindIII fragment obtained from pPLc24 and pPLc2819, respectively. Following ligation, the mixture was digested with BamHI to counterselect those molecules which had reconstituted the pPLc28 vector (pPLc28 contains a BamHI site between its EcoRI site and HindIII site (7)). Transformants were obtained in strain K514(λ) selecting for resistance to carbenicillin (100 μ g/ml).

Among 10 clones analysed, eight had acquired a single SalI site as predicted by joining the TaqI site in the multilinker sequence of pPLc2819 to the TaqI site succeeding the initiator ATG of the replicase gene. A representative plasmid, designated pPLc245, was linearized with either EcoRI or XbaI and produced two fragments after cleavage with PstI (data not shown). The nucleotide sequence around the ribosome binding site and initiating ATG of the replicase gene were verified by direct analysis using the Maxam and Gilbert method (15). pPLc245 DNA was cleaved at its unique EcoRI site and labelled by filling in of the sticky ends with DNA polymerase I (Klenow fragment) in the presence of α (³²P)ATP. After restriction with HaeII, the smaller of the labelled fragments was purified on acrylamide gel and its sequence determined (data not shown). The sequence of the relevant region of the plasmid is shown in Fig. 1.



Sequence at the ATG initiator codon:

AGGATTACCC **ATG** TCGACCCTCTAGAGCCTGCAGCCTCTAGAGCCAAGCTT
 SD SalI XbaI PstI XbaI HindIII

FIG. 1. Genetic and restriction map of expression plasmid pPLc245. The ampicillin resistance gene (Ap^R), the origin of replication and the P_L promoter were derived from pPLc28 (7). Only those restriction sites are shown which are unique in the plasmid.

In plasmid pPLc245, the ribosome binding region of the phage MS2 replicase gene is situated downstream from the powerful leftward promoter (P_L) of phage λ present on the plasmid. The G-residue of the initiating ATG codon constitutes the 3'-end of a Sall cleavage site. Thus, the initiating ATG of the replicase gene can be made accessible to blunt end ligation after cleavage with Sall and blunting of the 5'-sticky end with e.g., S_1 nuclease. In this manner, the coding region of any gene blunted by appropriate genetic engineering techniques can be joined to the initiating ATG of the replicase. The 3'-end of the inserted gene can be either a blunt end, an XbaI, PstI or HindIII site. Other applications include the sticky ligation of Sall or XhoI fragments into the unique Sall site. The XbaI sequence following the Sall site of pPLc245 contains a UAG stop codon in phase with the ATG of the replicase, thus precluding in-phase insertion at either the XbaI, PstI or HindIII sites. However, by filling in the Sall site and reclosing the plasmid, the UAG codon can be moved into another frame. As a consequence, a PvuI site is generated (this site occurs once more in the β -lactamase gene). Insertion of a suitable gene fragment in the above mentioned sites could then lead to the inducible synthesis of a fusion polypeptide with a very short extraneous N-terminal sequence.

2. Insertion of the human β -interferon gene into pPLc245

To test the usefulness of pPLc245 for expression of inserted genes, we cloned the sequence coding for mature human β -interferon (HFIF) (13) downstream from the initiating ATG of pPLc245.

Plasmid pPLc245 was digested with Sall and treated with S_1 nuclease. The DNA was further digested with HindIII and loaded on a 1.2% agarose gel. A band corresponding to linear pPLc245 was eluted from the gel. As a source of the coding region for mature human β -interferon, we used a recombinant DNA molecule into which an XbaI site had been engineered in front of the sequence coding for mature β -interferon (details of the construction of this plasmid (pPLcRX19) are described in ref. 16). In plasmid pPLcRX19, the sequence at the ATG codon of mature interferon (13) reads -TCTAGATG-. Following XbaI cleavage and S_1 nuclease treatment, the ATG codon of mature β -interferon can be made accessible as a 5' blunt end. In plasmid pPLcRX19, the coding sequence of β -interferon is followed by a HindIII site. Hence, plasmid pPLcRX19 was digested with XbaI, treated with S_1 nuclease and further digested with HindIII. A 520 bp fragment encompassing the complete coding region for β -interferon was eluted from the gel and ligated to the above mentioned Sall-, S_1 - and HindIII-treated sample of pPLc245. Transformants were obtained in strain K12 Δ H1 Δ trp selecting for resistance to carbenicillin (100 μ g/ml). To search for an in phase junction of the coding region for mature β -interferon to the ATG codon of the MS2 replicase, we made use of the

observation that even marginal expression of β -interferon led to a drastic reduction of the growth rate of the bacteria (see below). Using this simple screening method, we found one transformant among 32 tested which grew normally at 28°C but virtually failed to grow at 42°C. This clone harbored a plasmid which had lost the SalI site present in the acceptor plasmid pPLc245. The plasmid contained two PstI sites consistent with the insertion of the β -interferon gene into the vector pPLc245. One PstI site is present in the Ap^R-gene of pPLc245 and another is present in the coding region of β -interferon (13). The plasmid contained a single EcoRI and a single HindIII site. Combined digestion with EcoRI and HindIII produced a fragment slightly larger than an XbaI - HindIII fragment obtained from pPLcRX19 (see above), again consistent with the insertion of the β -interferon gene in pPLc245. This plasmid was designated pPLc245-HFIF25.

In order to verify the structure of pPLc245-HFIF25, plasmid DNA was cleaved with EcoRI, filled in at the staggered ends with α -³²P-ATP and digested with PvuII (a unique site present in the β -interferon coding region (13)). The smaller of the two labelled fragments was purified on a 6% acrylamide gel and subjected to sequence analysis according to Maxam and Gilbert (15). The sequence around the junction point between the blunted SalI site of pPLc245 and blunted XbaI site of pPLcRX19 reads: TGAGGATTACCCATGAGCTAC (data not shown). The construction scheme of pPLc245-HFIF25 predicted the presence of two ATG codons at the junction point; one derived from the initiator region of pPLc245 and one derived from the mature β -interferon sequence. The results show that one ATG codon was lost either from the blunted SalI site of pPLc245 or the blunted XbaI site of pPLcRX19, presumably due to overdigestion by the S₁ nuclease.

3. Synthesis of mature β -interferon

The synthesis of mature β -interferon in induced cultures of strain K12 Δ H1 Δ trp (pPLc245-HFIF25) was monitored by the appearance of a new protein and by detection of β -interferon activity in bacterial extracts. The cells were grown in LB medium at 28°C to a density of 2×10^8 /ml. An aliquot of the culture was then shifted to 42°C and samples were analysed on SDS-polyacrylamide gels. In the induced cells, a new major protein with an apparent molecular weight of around 19K was detected (Fig. 2). This protein was absent in induced cultures of the parent plasmid pPLc245. The observed molecular weight is consistent with the molecular weight of mature β -interferon calculated from sequence data (13).

In order to determine the amount of β -interferon synthesized, the cellular proteins were uniformly labelled. The cells were inoculated at a density of 10^6 /ml in LB medium containing 5 μ Ci/ml of ¹⁴C-amino acid mixture (The Radiochemical Centre, Amersham, England) and grown at 28°C to a density of 2×10^8 /ml. Following an induction period of 180 min at

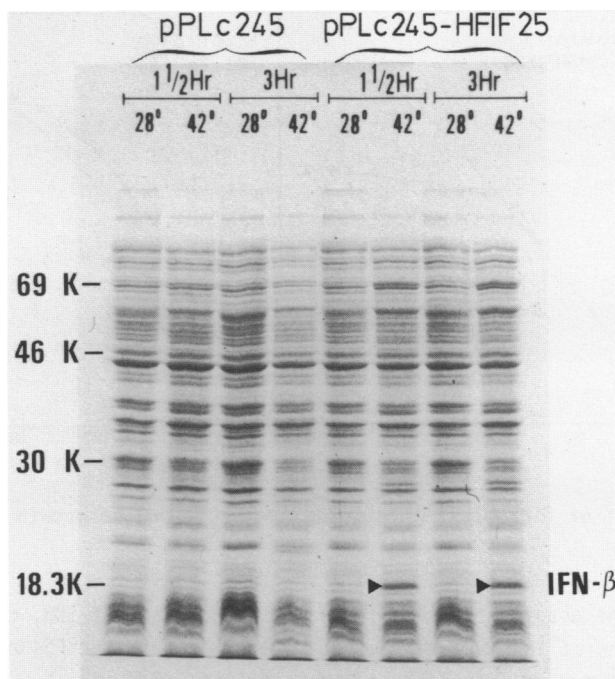


Fig. 2. Bacterial synthesis of fibroblast interferon.

Cultures of strain K12ΔH1Δtrp containing either pPLc245 or pPLc245-HFIF25 were grown and thermo-induced as described in the text. At 90 min and 180 min after induction, aliquots were collected, boiled in SDS and electrophoresed in a 12.5% polyacrylamide gel. The gel was stained with Serva blue R250. Each lane contains the equivalent of 1×10^8 cells. The interferon band is shown by a triangle. As molecular weight standards, we used a set of methylated ^{14}C -labelled proteins (New England Nuclear, Boston, Mass.).

42°C , the cellular proteins were separated on SDS-polyacrylamide gels and the radioactivity incorporated in individual protein bands was determined. Under the conditions used, it can be assumed that the incorporated radioactivity reflects the actual amount of protein present in the cell. The induced 19K protein was found to account for 2% of total cell protein. This corresponds to about 100,000 molecules per cell.

The biological activity of the bacterially made interferon was assayed as described in Materials and Methods. Using this procedure, we found that strain K12ΔH1Δtrp (pPLc245-HFIF25), induced for 180 min, produced an interferon titre of between $2\text{--}6 \times 10^9$ international units per litre of culture at a cell density of $4 \times 10^8/\text{ml}$. Uninduced cultures contained less than 3×10^4 units per litre (this value represents the limit of

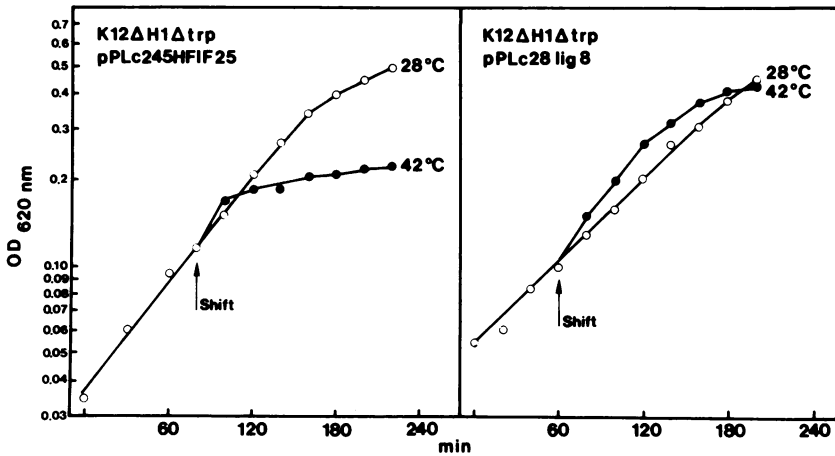


Fig. 3. Effect of fibroblast interferon synthesis on the growth rate of *E. coli*.

detection in the assay system used; due to the presence of SDS, the first serial dilutions of the bacterial extracts are toxic to the FS4 cells).

4. Influence of interferon synthesis on host cell metabolism

Figure 3 shows the growth rate of the *E. coli* strain K12ΔH1Δtrp synthesizing either human β -interferon or phage T₄ DNA ligase (11). Remarkably, the synthesis of interferon which is quantitatively less than that of ligase (2% versus 10% of total cellular protein) drastically reduces the growth rate of the bacteria. The effect was observed in several bacterial strains. In order to study interferon synthesis directed by pPLc245-HFIF25 in *E. coli* strains which do not harbor a defective λ prophage, these strains were first transformed with pcI857. This plasmid is compatible with ColE1-derived replicons and codes for a temperature sensitive repressor (11). We found that the amount of β -interferon accumulating in the cells after induction differed greatly, depending on the strain tested. The results are summarized in Table II. Although, e.g., strain MC1061 (pcI857) (pPLc245-HFIF25) accumulates interferon to only one fifth of the value found for strain K12ΔH1Δtrp, its growth rate is still severely inhibited after induction. It should be noted that we have not measured actual rates of synthesis but merely accumulation of the product after a 3 h induction period. It is possible that the synthesis rate does not differ greatly, but rather that the interferon is degraded differentially in the several strains. In favor of this hypothesis is the observation that a *deg* strain (17), which is impaired in its proteolytic degradation system, allows accumulation of the

Table II Synthesis of β -interferon in different strains containing pPLc245-HFIF25

<u>Strain</u>	<u>% of total protein</u> ^(a)	<u>IFN-activity</u> ^(b) <u>units/L culture</u>
K12 Δ H1 Δ trp	2%	2×10^9
M5219	ND	2×10^7
MC1061 (pcI857)	0.4%	4×10^8
SG4044 (pcI857)	4%	ND

a) the total amount of protein synthesized was determined as described in the text; b) the cell density of the cultures at the time of induction was 4×10^8 /ml; the induction period was 3 h; ND = not determined

product up to 4% of total cell protein. The latter strain is particularly well suited for large scale production of interferon. Unlike strain K12 Δ H1 Δ trp, which for an unknown reason cannot be grown to very high densities, strain SG4044 can do so in rich media and under conditions of forced aeration.

The very low interferon titres found in strain M5219 are unlikely to be due solely to increased proteolytic breakdown, since the strain is isogenic with K12 Δ H1 Δ trp. Upon induction, strain M5219 synthesizes an active N-gene product from the defective prophage. The N-protein in conjunction with a nut site is known to function as an antiterminator for transcription initiated at P_L or P_R (18). We have previously shown that prolonged induction of pPLc plasmids in M5219 leads to curing of the plasmid (7). However, analysis of plasmid DNA of induced cells shows that the curing is not rapid enough to account for the much lower interferon titre in this strain (data not shown). It is possible that a long untranslated 3'-end of the message renders it more susceptible to nucleolytic breakdown or, through structural effects, less efficient for initiation of translation.

DISCUSSION

We have previously described the use of the P_L promoter of phage λ , cloned on plasmids, for efficient transcription of downstream gene sequences. The system allows full control over the expression of the gene by making use of a temperature-sensitive repressor, product of the cI857 gene supplied in trans from a resident defective prophage or from a compatible plasmid (6,7,11). However, these vectors did not contain an

easily accessible ribosome binding site. The expression of an inserted gene depended, therefore, on the presence and the functionality in E. coli of its own ribosome binding site.

In this paper we describe the incorporation into PL-vectors of a ribosome binding site in such a way that the ATG codon can easily be made accessible for insertion of any coding sequence. For this purpose, we decided to use the ribosome binding site and ATG codon of the replicase gene of the RNA phage MS2. The rationale for this choice was based on the observation that the MS2 replicase cloned downstream from the P_L promoter was induced to very high levels in the cell (10). Similarly, a fusion polypeptide between an amino-terminal fragment of MS2 replicase and the VP1 protein of Foot and Mouth Disease Virus was produced in large amounts in E. coli (19). To expose the initiating ATG codon of the replicase, we transformed a TaqI site right after the ATG into a unique Sall site. After cleavage with Sall and resection of the 5' protruding end with, e.g., S₁ nuclease, the ATG-codon is accessible as a blunted end. In plasmid pPLc245, the Sall site is followed by the sequence: XbaI - PstI - XbaI - HindIII. Except for the PstI site which occurs once more in the Ap gene, these sites are unique in the plasmid. Any coding region blunted at the 5'-end can thus be inserted in phase with the initiating ATG of the replicase; the 3'-end of the inserted fragment can be a blunt end or an XbaI, PstI or HindIII end.

To test the effectiveness of pPLc245, we inserted the coding region for mature human fibroblast β -interferon downstream from the ATG of MS2 replicase. Induced cultures containing pPLc245-HFIF25 accumulated the protein to about 2%-4% of total cellular protein depending on the host strain, as determined by measuring actual protein content. The induced protein was easily detected by staining of cellular proteins electrophoresed in polyacrylamide gels. The product was biologically active as determined by a cytopathic effect-inhibition assay. Titres of $2-6 \times 10^9$ international units per litre of culture at a cell density of 4×10^8 /ml were obtained. This value is considerably higher than the titres reported previously for human β -interferon expressed under the control of the E. coli lac promoter (20,21) or the E. coli trp promoter (21,22). Assuming a specific biological activity of 4×10^8 IU/mg (23), we calculated the interferon to be present at about 400,000 molecules per cell. From the determination of actual protein content we found a value of about 100,000 molecules per cell. These two values are in reasonable agreement with each other. The comparison shows that our method of extraction is not harmful to the protein.

The amount of biologically detectable interferon in uninduced cells was at least five orders of magnitude lower than the amount found after a three hour induction period. It is unclear whether this difference is

caused by the sole action of repressor. Indeed, uninduced basal levels of trpA gene cloned on PL-plasmids were only three-hundred fold lower than the induced level (7). One possibility is that the small amount of protein which is produced at 28°C is much more liable to be degraded by the host's proteases. Stabilization of intrinsically unstable proteins by overproduction in *E. coli* has been noted, e.g., in the case of a nonsense fragment of β -galactosidase (24).

Remarkably, bacterial cultures containing pPLc245-HFIF25 almost ceased to grow after shift-up to 42°C. This is a rather specific effect, neither observed with the vector itself, nor with a similar vector synthesizing large amounts of plasmid-coded T₄ DNA ligase (11). We do not know whether the effect is a direct or indirect consequence of the synthesis of interferon, nor have we studied at what level the synthesis of interferon interferes with bacterial metabolism. Possibly, it may be related to the fairly hydrophobic nature of β -interferon which causes the protein to stick to the bacterial membrane. In any case, this observation stresses the importance of being able to regulate the expression of a cloned gene. We observe that a bacterial strain containing pPLc245-HFIF25 grows quite normally at 28°C under non-expressing conditions, but is severely impaired in its growth rate at 42°C when the inserted gene is maximally expressed. As a consequence, it would appear that a host-vector system which does not allow full control over the expression of an inserted gene will tend to be unstable and will have a negative selection value. In pPLc245, the expression of an inserted gene can be completely repressed by propagating the culture at 28°C. Full scale production of a gene product is subsequently obtained at 42°C in a terminal phase of the culture.

ACKNOWLEDGEMENTS

We thank R. DERYNCK for his contribution in the construction of several intermediate plasmids to arrive at pPLcRX19; F. DUERINCK for DNA sequence determination; W. BURMS for performing the interferon assays; and M. BENSCH and R. WULGAERT for technical assistance. F. SHAPIRO and W. DRYVERS were very helpful in editing the manuscript and taking care of the artwork. This research was supported by BIOGEN, S.A. and by a grant from the Gekoncerteerde Onderzoeksakties of the Belgian Ministry of Science.

REFERENCES

1. Backman, K., Ptashne, M. and Gilbert, W. (1976) Proc. Natl. Acad. Sci. USA 73, 4174-4178.
2. Backman, K. and Ptashne, M. (1978) Cell 13, 65-71.
3. Fuller, F. (1982) Gene 19, 43-54.
4. Hallowell, R.A. and Emtage, S. (1980) Gene 9, 27-47.
5. Tacon, W., Carey, N.H. and Emtage, S. (1980) Mol. Gen. Genet. 177, 427-438.

6. Bernard, H.-V., Remaut, E., Hershfield, M.V., Das, H.K., Helinski, D.R., Yanofsky, C. and Franklin, N. (1979) *Gene* 5, 59-76.
7. Remaut, E., Stanssens, P. and Fiers, W. (1981) *Gene* 15, 59-76.
8. Shimatake, H. and Rosenberg, M. (1981) *Nature* 292, 128-132.
9. Gold, L., Pribnow, D., Schneider, T., Shinedling, S., Singer, B.S. and Stormo, G. (1981) *Ann. Rev. Microbiol.* 35, 365-403.
10. Remaut, E., De Waele, P., Marmenout, A., Stanssens, P. and Fiers, W. (1982) *The EMBO Journal* 1, 205-209.
11. Remaut, E., Tsao, H. and Fiers, W. (1983) *Gene*, in press.
12. Thuring, R.W.J., Sanders, J.P.M. and Borst, P. (1975) *Analytical Biochemistry* 66, 213-220.
13. Derynck, R., Remaut, E., Saman, E., Stanssens, P., De Clercq, E., Content, J. and Fiers, W. (1980) *Nature* 287, 193-197.
14. Fiers, W., Contreras, R., Duerinck, F., Haegeman, G., Iserentant, D., Merregaert, J., Min Jou, W., Molemans, F., Raeymakers, A., Vandenberghe, A., Volckaert, G. and Ysebaert, M. (1976) *Nature* 260, 500-507.
15. Maxam, A. and Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* 75, 560-564.
16. Stanssens, P. (1983) Ph.D. thesis, State University of Ghent, Belgium.
17. Gottesman, S. and Zipser, D. (1978) *J. Bact.* 133, 844-851.
18. Salstrom, J.S. and Szybalski, W. (1978) *J. Mol. Biol.* 124, 195-221.
19. Kupper, H., Keller, W., Kurz, C., Forss, S., Schaller, H., Franze, R., Strohmaier, K., Marquardt, O., Zaslavsky, V.G. and Hofschneider, P.H. (1981) *Nature* 289, 555-559.
20. Taniguchi, T., Guarente, L., Roberts, T., Kimelman, D., Douhan, J.III., and Ptashne, M. (1980) *Proc. Natl. Acad. Sci. USA* 77, 5230-5233.
21. Goeddel, D.V., Shaepard, H.M., Yelverton, E., Leung, D. and Crea, R. (1980) *Nucleic Acids Research* 8, 4057-4074.
22. Shepard, H.M., Yelverton, E. and Goeddel, D.V. (1982) *DNA* 1, 125-131.
23. Knight, E.Jr. (1976) *Proc. Natl. Acad. Sci. USA* 73, 520-523.
24. Cheng, Y.E., Kwok, D.Y., Kwok, T.J., Soltvedt, B.C. and Zipser, D. (1981) *Gene* 14, 121-130.
25. Zabeau, M. and Stanley, K. (1982) *The EMBO Journal* 1, 1217-1224.
26. Casadaban, M.J. and Cohen, S.N. (1980) *J. Mol. Biol.* 138, 179-207.