The effects of DNA sequence on interferon mRNA biosynthesis and stability in Ecoli

A thesis submitted in accordance with the requirements of the University of London for the Degree of Doctor of Philosophy.

by

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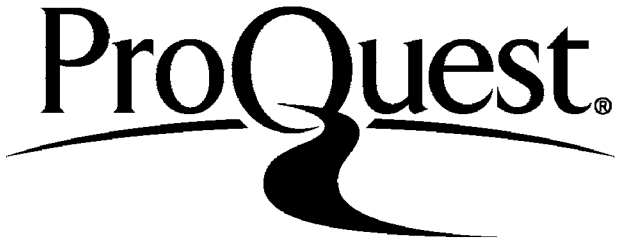

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ABBREVIATIONS

In general the abbreviations and symbols employed in this thesis are those described in the Instructions to Authors for the Biochemical Journal (Biochem. J. (1978) 169, 1-27).

Construct	Bacterial strain containing the plasmid
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
Tris	Tris (hydroxymethyl) aminomethane
mRNA	messenger ribonucleic acid
SDS	sodium dodecyl sulphate
IAA	3β-indole acrylic acid
TBE	Tris-borate electrophoresis buffer
TAE	Tris-acetate electrophoresis buffer
EE	Electroelution buffer
EDTA	ethylaminediaminetetracetic acid
TEMED	N, N, N', N'-tetramethylethylenediamine
mwt	molecular weight
trp	tryptophan
dATP	deoxyriboadenosine 5'-triphosphate
dCTP	deoxyribocytisine 5'-triphosphate
dGTP	deoxyriboguanosine 5'-triphosphate
dTTP	thymidine 5'-triphosphate
ATP	adenosine 5'-triphosphate
amp	ampicillin
tet	tetracycline
M9CA	M9 minimal salts medium plus casamino acids
MOPs	Potassium morpholinpropane sulphate
E.coli	Escherichia coli

IPTG	isopropyl-β-D-thio-galactopyranoside
X-Gal	5-bromo-4-chloro-3-indolyl-β-galactoside
Tricine	N'-Tris (hydroxymethyl) methyl glycine
PEG	polyethylene glycol
DPC	diethyl pyrocarbonate
(rC)	ribocytodine
ddATP	dideoxyriboadenosine 5'-triphosphate
ddCTP	dideoxyribocytisine 5'-triphosphate
ddGTP	dideoxyriboguanidine 5'-triphosphate
ddTTP	deoxythymine 5'-triphosphate

Abstract

The aim of this investigation was determine the effect of DNA sequences on interferon mRNA biosynthesis and stability in E.coli.

Interferon α1 and α2 DNA sequences are 80% homologous. Despite this large degree of similarity different levels of protein and different transcription products were obtained when the two genes were cloned into identical vectors. Increasing the plasmid copy number and introducing a transcription terminator immediately downstream from the interferon sequence increased α1 production. Deleting the sequence upstream from the -35 region of the promoter or reducing the length of the 3' non-coding region had little affect on α2 production. Highest levels of α2 interferon were obtained by altering the ribosome binding site. This necessitated decreasing the plasmid copy number to achieve maximum plasmid stability. In contrast, all α1 containing plasmids were stably maintained in the bacteria.

Analysis of the transcription products of all the interferon producing clones revealed that multiple mRNA species were generated by all the constructs except those which contained the termination signal. Removal of the sequence 5' to the -35 region of the promotor resulted in reduced transcription, while shortening the 3' non-coding region lowered termination efficiency within the remaining sequence facilitating the production of polycistronic messages. Stability studies were undertaken to determine the half-lives of the mRNA species. Most decayed with a half-life of 1-1.5 minutes. However, more stable species were produced by the constructs containing the terminiation signal.

Comparison of the mRNA species produced by similar α1 and α2 producing strains revealed that while all the α1 and α2 mRNA were of sufficient length for translation to yield mature interferon three α2 mRNA species appeared too short. These prematurely terminating mRNA were further characterized. The smallest terminated at a rho-dependant

terminiation site while the other two species terminated at rho-independent sites. Termination at the rho-dependant site could be eliminated if the rare arginine codons AGG present in the early coding sequence were replaced by the more commonly used arginine codons CGT. Transcription profiles and mRNA half-lives were determined in different media at different growth rates to see whether production of the prematurely terminating species was affected. While all species were produced under all conditions the distribution of mRNA species altered with both media and growth phase.

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(ii) 367 base pair fragment

(iii) 302, 88, 172, and 114 base pair fragments

(iv) 815 base pair fragment

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I: INTRODUCTION

A. Introduction:

The refinement of genetic engineering techniques has enabled the production of clinically desirable proteins that are difficult to purify in sufficient quantities by conventional methodology. The gene encoding the required protein is isolated from its natural source and cloned into a system under appropriate control signals that enable protein production. Using recombinant DNA technology large quantities of interferons that are difficult to purify from natural sources have been obtained.

B. Requirements for efficient expression of eucaryotic genes in E.coli:

To enable expression of eucaryotic genes, such as interferon, in bacterial systems two absolute requirements must be met. Firstly, a bacterial promoter must be placed immediately upstream from the inserted sequence to facilitate transcription. Secondly, a ribosome binding site must be present to enable the ribosomes to bind and initiate translation. The ribosome binding site may be produced by joining the eucaryote gene to the beginning of a bacterial gene to create a fused polypeptide product (Itakura et al. 1977, Mercereau-Puijalon et al. 1978, Villa-Komaroff et al. 1978 and Emtage et al. 1980). Alternatively, the ribosome binding site may be formed by construction of a hybrid site by combining a bacterial "Shine-Dalgarno" sequence (Shine and Dalgarno, 1975) with the translation initiation codon inserted at the beginning of the eucaryote gene. In this case either a normal eucaryotic protein or one containing an additional N-terminal methionine is produced (de Boer et al, 1983, Goeddel et al, 1981, Jay et al, 1982 and Looman et al. 1985). Unless production of a fused protein is necessary to increase yields or to reduce toxicity use of a hybrid ribosome binding site is preferred as it enables the direct isolation of the mature eucaryotic gene product.

Factors known to affect the efficiency of gene expression include choice of promoter, the sequence of bases in the vicinity of the ribosome binding site, the plasmid copy number and the presence of transcription terminators (Carrier et al. 1983). For high levels of expression, transcription should be initiated from a strong inducible promoter. Inducibility is important because constitutive expression will almost inevitably be detrimental to the host. Efficient translation depends also on ribosome binding and recognition of the translation initiation codon at the start of the gene sequence. The ribosome binding site is deemed to be the region protected by the ribosome from nuclease digestion (Steiz, 1979). Within this site is a region known as the "Shine-Dalgarno" region (Shine and Dalgarno, 1974) which is complementary to the 5' end of the 16S ribosome RNA, usually 4-9 bases in length and essential for efficient translation. The spacing between the "Shine-Dalgarno" sequence and the initiation codon is known also to affect the level of translation (Shepard et al. 1982). The preferred distance between these two regions is 5-9 nucleotides. The base composition immediately 5' and 3' to the initiation site (Schwartz et al. 1981 and Hui et al. 1984) and regions more external to the ribosome binding site (Kastelein et al. 1983) can also affect translation.

Most commonly used cloning vectors are based on the multicopy ColE1-related plasmids such as pBR322 and pAT153. Under normal conditions these two plasmids are present in 30-50/90-150 copies per chromosome equivalent respectively. Increasing plasmid copy number increases the number of RNA transcripts of the gene available for binding to the ribosome. Since ribosome/mRNA binding is the rate-limiting step in translation any process that increases the number of transcripts avaliable for binding must increase the rate of translation.

The use of transcription terminators to minimise the length of the recombinant transcript was found to be an important consideration when

constitutive or highly efficient promoters were used to drive transcription (Gentz et al. 1981)

The effects of changing DNA sequences on RNA transcription, the coupling of transcription to translation and mRNA stability are discussed in the following sections.

1. Transcription and DNA sequences affecting levels of mRNA synthesis:

Transcription is the process whereby genes are selectively located, recognised and transcribed by a DNA-dependent RNA polymerase to produce RNA. This process is regulated at each phase by a complex series of interactions between the polymerase, the DNA, the nascent RNA and a variety of small regulatory molecules. The process can be divided into four phases; polymerase location and recognition of the promoter, initiation of RNA synthesis, elongation of the RNA transcript and termination and release of the nascent RNA and polymerase from the DNA template.

(a) Components of the transcription system:

(i) RNA polymerase:

The enzyme central to transcription, RNA polymerase, catalyses the transfer of a ribonucleoside monophosphate to the 3'-OH of the growing RNA chain using as substrates, ribonuoleoside triphosphates. It was discovered in 1959 by Weiss and Gladstone. The E.coli enzyme consists of a core, comprising two α subunits (mwt 36,512), a β subunit (mwt 150,819) and a β' subunit (mwt 155,162) (Chamberlin, 1982). The core plus another subunit, sigma (mwt 70,236) constitute the holoenzyme which is responsible for recognition of and initiation at the promoter site. Elongation is directed by the core enzyme alone.

The DNA-dependent RNA polymerase is able to distinguish promoter sequences from similar non-promoter sequences along the length of the double stranded DNA and selectively bind to these regions. Once bound, the polymerase transcribes the RNA releasing the nascent RNA transcript and

template DNA only when appropriate termination signals are reached. The polymerase recognises regulatory sites on both DNA and RNA interacting with protein factors and small molecules that can modulate the enzymic activity. (von Hippel et al. 1984.)

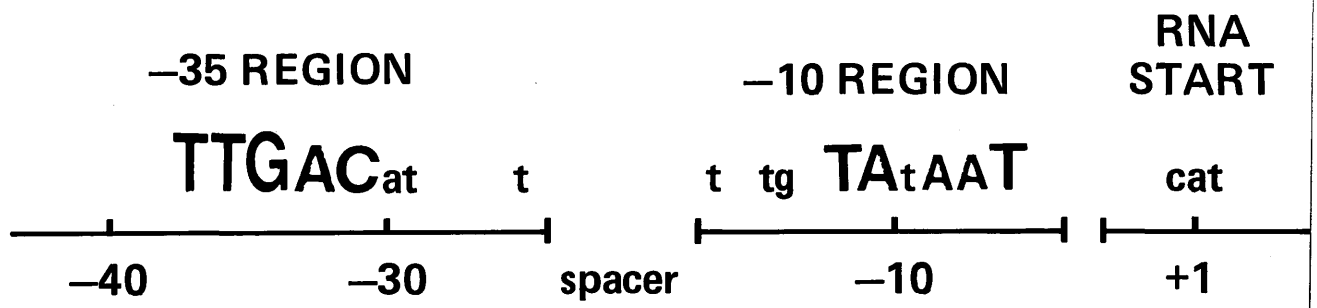
When the polymerase is bound to the promoter, 75 base pairs are protected by the enzyme from nuclease digestion (Galas and Schmitz, 1978) compared with only 30 base pairs protected when the polymerase is in the elongation mode (von Hippel et al 1984). The β subunit has been shown to be involved in the binding of nucleotide substrates while the β' subunit is involved in DNA binding. Both the β and β' subunits contain a single atom of Zn^{2+}. (Speckhard et al. 1977, Wu et al. 1977 and Millar et al. 1979.) No specific role for the α subunit has been found. The constituents of the core enzyme are individually enzymatically inactivate and incapable of binding to DNA (Chamberlin, 1982).

(ii) Promoters:

The promoter is the DNA sequence recognised by the RNA polymerase. It is generally defined as "the segment of DNA that contains signals that direct the proper binding of the RNA polymerase holoenzyme and its subsequent activation to a form capable of initiating specific RNA transcription." (von Hippel et al. 1984.)

Sequence analysis of more than 100 promoters from E.coli have been reported (Hawley and McClure 1983). The homologies observed in these sequences have been used to formulate a consensus sequence (Figure 1). There are three highly conserved bases, TTG in the -35 region and TA---T near the -10. There are also areas of sequence that are weakly conserved on either side of the -35 and -10 regions. It appears that the consensus sequence represents maximal promoter strength as mutations that decrease homology to the consensus are down mutations

Figure 1: E.coli promoter consensus sequence:



The sequence homologies at positions within the promoter are shown in three classes. The highly conserved bases (>78%) are shown in large capital letters. The conserved bases (>50%) are shown in small capital letters. The weakly conserved bases (>40%) are shown in lower case letters. The distance betwen the -38 and -10 regions is ordinarily 17+/- 1 base pair.

Mc Clure (1985).

while those that increase homology are up mutations. A semisynthetic promoter, tac, containing the -35 region of the trp promoter and the -10 region of the lac UV5 promoter (de Boer et al. 1983) is very close to the consensus sequence and was found to promote RNA chain initiation at very high frequencies both in vivo and in vitro. No wild-type E.coli promoters have been found that match the consensus sequence, even in the highly conserved regions, suggesting that promoter function is optimised and not maximised in vivo.

The location of promoters may also be important in transcription initiation. More than one third of the promoters studied by Hawley and McClure (1983) were close to other promoters. They could be tandemly arranged and transcribed in the same direction, (eg the P1 and P2 sites in the gal promoter, Adhya and Gottesman (1982)) divergently orientated enabling two polymerase molecules to bind within a common control region and transcribe in opposite directions (eg the ampicillin and tetracycline genes of pBR322) or convergently orientated. This latter class of promoters occur infrequently and in these cases the polymerase molecules oppose one another and transcribe both strands of the DNA over a common interval (Simons and Kleckner, 1983). Promoters may compete for polymerase binding if they are in too close a proximity to each other. This phenomenon is termed promoter occlusion (Adhya and Gottesman, 1982.)

Several external factors have been shown to regulate promoter strength. These include DNA structure, DNA supercoiling and ancillary proteins such as repressors and activators. At saturating concentrations of activator promoter strength is maximal while at saturating concentrations of repressor promoter strength is minimal.

Structural and conformational changes to the DNA template that have been shown to affect promoter strength include the deletion of DNA 50-80 base pairs upstream of the E.coli tRNA tyr promoter. Deletion of these

sequences decreased promoter strength (Lamond and Travers. 1983). Deletion of segments of DNA sequence containing A+T rich regions upstream of the -35 region of the trp promoter resulted in decreased promoter strength (Nishi and Itoh, 1986).

Supercoiling of the DNA has been shown in vivo and in vitro to have direct effects on certain promoters. However, both increased and decreased gene expression has been observed. (McClure, 1985.)

(b) Recognition and location of the promoter by RNA polymerase:

Promoters are recognised by RNA polymerase as double-stranded sequences. The base-pair sequences are identified as specifically orientated hydrogen bond donors and acceptors via the "grooves" of the DNA double helix. These hydrogen bond clusters are recognised by a complementary cluster of hydrogen bonds comprising the binding domain of the polymerase. Very little is known about the regions of the polymerase that may be involved in this interaction. It is known that the sigma factor is involved in promoter recognition and that the α,β and β' subunits may be cross-linked to DNA under various conditions. (Miller et al. 1979.)

The binding of RNA polymerase to nonpromoter DNA is essential in promoter location. (von Hippel et al. 1974, Winter et al. 1981.) It has been hypothesised that the RNA polymerase slides along the DNA until it comes into contact with and binds to promoter sequences. (von Hippel et al. 1984.) This hypothesis requires that the polymerase exists in more than one binding conformation and that these conformations be interchangable.

The RNA polymerase bound to the double stranded DNA promoter is said to be in a closed promoter complex. Evidence for the existence of such a species comes from kinetic analysis of RNA synthesis which shows a lag phase with double stranded but not single stranded DNA templates. (Walter et al. 1967, McClure, 1980.) The promoter must form a single stranded region of contact with the polymerase in order to synthesize mRNA. This is termed the open complex.

Chemical and enzymic analysis of the promoter-polymerase interaction have revealed that the -35 and -10 regions are substantially protected by the polymerase in the bound state. (Siebenlist et al. 1980, von Hippel et al. 1984.) These protected sites are located largely on one side of the DNA suggesting that the polymerase may interact effectively with either the double or single stranded DNA.

(c) Initiation of RNA synthesis:

The binding of the first ribonuoleoside triphosphate to the open-polymerase complex signals the commencement of RNA synthesis. Transcription in E.coli is initiated almost exclusively at purine nucleotides. A recent survey of 88 promoters revealed that 51% intiate transcription at an A residue, 42% at G, 5% at C and only 2% at a T residue. (Hawley and McClure, 1983.) The location of the initiating nucleotide within the promoter is strongly correlated with the positions of the -10 and -35 regions.

The binding of the nucleoside triphosphates to the polymerase is an ordered process with the initiating NTP binding first. The affinity of the polymerase for this nucleotide is much greater than for subsequent ones and it has been suggested that some of the free energy of binding is used to drive the polymerase into an initiation conformation. (von Hippel et al. 1984.)

RNA polymerase is a large enzyme which can protect approximately 65 base pairs of DNA from enzymatic digestion when bound in the open complex. (von Hippel et al. 1984). For maximum initiation frequency at the promoter the polymerase molecule must move out of the promoter region so that other polymerase molecules may bind and initiate further RNA synthesis. Several factors have been shown to influence promoter clearance time. Abortive initiation has been shown to occur in vitro even in the presence of all four nucleotides. This process has been quantitated for the lac UV5 and Tn5 promoters (Munson and Reznikoff, 1981, Stefano and Gralla, 1979) and it is likely that it contributes significantly to the overall frequency of long chain

synthesis in the case of the lac UV5 promoter. A second factor influencing promoter clearance involves RNA polymerase pausing near the start site. A third event, the release of the sigma factor defines the end of the initiation phase and the commencement of the elongation phase.

Using the maximum elongation rate of 30-50 nucleotides per second it takes a minimum of 1-2 seconds for RNA polymerase to clear the promoter. The transition between the open complex and the transcribing complex produces changes in the enzyme-RNA and enzyme-DNA contacts as transcription proceeds. (McClure, 1985.) Once a certain number of phosphodiester bonds are formed the polymerase-RNA-DNA complex becomes more stable as shown by an increased resistance to dissociation by high salt, resistance of the polymerase to heat denaturation, increased resistance of polymerase to digestion by trypsin and resistance to inhibition by rifampicin (von Hippel et al. 1984).

(d) Elongation:

The elongation reaction results in the addition of a nucleotide to the 3'-OH end of the nascent RNA chain and subsequent translocation of the polymerase along the DNA template by one base pair. The movement of the polymerase involves the "melting" of the DNA template duplex in addition to displacement of the 5' end of the nascent RNA-DNA hybrid. The unwinding and rewinding of the DNA template and the displacement of the 5' end of the RNA-DNA hybrid are catalysed by the elongating polymerase. (Gamper and Hearst, 1982.)

Elongation rates are not uniform. Studies of several natural DNA templates have shown sequences where elongation is very slow compared to the average. These positions are called pausing sites. (Kassavetis and Chamberlin, 1981, Reisbig and Hearst, 1981, Morgan et al. 1983b.) Pausing occurs at these sites, even in the presence of high concentrations of the four nucleotides, indicating that the effect is template sequence-dependent.

Sequence features that appear to produce pausing are the presence of GC-rich regions approximately 10 base pairs upstream of the paused transcript and the presence of dyad symmetry elements centered 16-20 base pairs upstream of the 3' end. (von Hippel et al. 1984.)

Several pausing sites that lack strong secondary structure are preceeded by regions that are GC-rich. The presence of such regions within the 5' half of the RNA-DNA hybrid has been postulated to interfere with the progress of the polymerase by resisting unwinding of the hybrid and thus obstructing reformation of the DNA duplex. (Kingston and Chamberlin, 1981, Morgan et al. 1983b, Lau et al. 1983.)

Extrinsic factors that have been shown to affect elongation are ppGpp and the Nus A protein of E.coli. Both factors induce polymerase pausing (von Hippel et al. 1984).

(e) Termination:

Transcription termination requires the cessation of elongation, release of the transcript from the ternary complex and dissociation of the polymerase from the template. Termination is determined by competition between two alternate irreversible pathways, elongation and disassociation of the ternary complex. Therefore, it is influenced by both the sequence and factor dependence of elongation rates. Two types of termination sites have been identified, one factor independent (rho-independent) and the other factor dependent (rho-dependent). Factors other than rho have also been shown to modulate termination. These include the N and Q genes of bacteriophage lambda and E.coli nusA protein (von Hippel et al. 1984).

(i) Rho-independent termination:

Termination sequences that function with purified RNA polymerase and without additional factors are called rho-independent terminators. Many such sites have been identified and a consensus sequence established (Rosenberg and Court 1979). The consistency of structure of these sites has

facilitated the development of a model for the process of rho-independent termination.

The rho-independent signal is contained within the DNA sequence but termination occurs as a result of secondary structure formation in the transcribed RNA and weak RNA:DNA interactions. The transcript must contain a region of stable GC-rich dyad symmetry enabling an RNA stem-loop structure of approximately 7 base pairs to form upstream of the 3' terminus and a stretch of several consecutive uridine residues. Termination occurrs within,or just distal to this oligo(rU) sequence. It is proposed that the dyad symmetry gives rise to the formation of a stem-loop structure in the nascent RNA, causing the transcribing polymerase to pause and thus disrupting the 5' half of the RNA-DNA hybrid. The remainder of the hybrid containing the oligo(rU) is unstable and melts, leading to the release of the transcript and reformation of the double stranded DNA template. The melting of the rU.rA is due to its unusual instability. (Platt 1981, Martin and Tinoco 1980.)

Variations in efficiency of rho-independent termination have been investigated with respect to dyad symmetry and oligo(rU) sequences (Christie et al. 1981). These experiments demonstrated that termination efficiency increased with increased length of the RNA stem-loop structure (from a minimum of six base pairs) and increased length of the uridine sequence. Incorporation of base analogues into either the nascent transcript or the template DNA confirmed the importance of the rU.rA base pair instability. (Farnham and Platt 1982, Neff and Chamberlin 1978.)

The importance of RNA polymerase pausing in rho-independent termination has been shown by the response to termination at the trp operon attenuator with rifampicin resistant polymerases with mutant,β subunits. The efficiency of termination by the mutant correlated to the extent of pausing by the polymerase. (Fisher and Yanofsky 1983 a&b.)

It is possible to conceive of a situation where the sequence preceeding the region of dyad symmetry consists of consecutive A residues. In these cases if the non-template DNA strand were to be transcribed a rho-independent terminator would be generated at this position. The DNA sequence provides a potentially bidirectional termination signal. Examples of such sites do exist. Bidirectionally active termination signals are located between the tetA and orfL genes on the transposon TnlO (Schollmeier et al. 1985,) and the phenylalanine and tyrosine operons (Hudson and Davidson 1984).

(ii) Rho-dependent termination:

When in vivo and in vitro transcription products were compared, in many cases the in vivo termination signals were not recognised by purified RNA polymerase. A protein factor, rho, that facilitates the accurate termination of transcription at specific sites in bacteriophage lambda was discovered by Roberts (1969). Subsequent work revealed that rho causes termination at many bacterial and phage sites and is responsible for translational polarity. (Adhya and Gottesman 1978.) It has been suggested that rho is esssential for cell growth (Das et al. 1976). Indeed, mutants that contain a temperature sensitive rho protein are unable to sustain growth at 42^{o}C. (Inoko et al. 1976.)

Biochemical investigations of the properties of rho have shown that it possesses a single-stranded RNA-dependent nucleoside triphosphohydrolase activity (NTPase). (Lowery-Goldhammer and Richardson 1974. Lowery and Richardson 1977a&b.) Hydrolysis of nucleoside triphosphates, usually ATP, is necessary for rho-dependent termination. (Howard and de Crombrugghe 1976.) Although it is known that rho binds to the nascent RNA the nucleic acid sequences at rho-dependent sites are heterologous and lack consensus sequences such as those found at rho-independent sites.

The rho protein is comprised of six identical subunits that aggregate at moderate concentrations to form a hexamer. The hexameric form is

stabilised by binding to RNA and by the presence of ATP. (Finger and Richardson 1982.) Twelve to fourteen residues of DNA are bound per monomer of rho (von Hippel et al. 1984) indicating that 72-84 residues are bound per rho hexamer.

Rho NTPase activity requires single-stranded RNA as cofactor. Double-stranded RNA is inactive, while NTP hydrolysis is stimulated under conditions that destabilise RNA secondary structure. (Richardson and Macy 1981.) Poly (rC) has the highest rho binding affinity and cofactor activity but other copolymers containing as little as 5-10% cytidine residues are also very active. (von Hippel et al. 1984.) Thus while the binding of rho depends upon the extent of single-stranded RNA regions, NTPase activity may depend upon the presence of a certain number of cytidine residues. (Richardson and Macy 1981.)

It appears that more than one rho nucleic acid binding site is required to activate NTP hydrolysis. Oligomers (rC)6-10 cooperatively stimulate NTPase activity as do poly(dC) and oligo(rC). (Richardson 1982.) However, poly(dC) by itself has no NTPase cofactor activity (von Hippel et al. 1984). These results show that multiple sites are involved but it may be that there are one or two classes of sites. In the simplest case single-stranded RNA may bind cooperatively to all the sites. Alternatively, rho may bind to the nascent RNA and additionally to either the noncoding DNA strand (Richardson 1982) or the 3' terminus of the transcript (Sharp et al. 1983). Recent evidence has shown that cooperative interactions between rho subunits are essential its for function. Richardson and Ruteshouser (1986) have shown that a mutant rho factor defective in interactions with RNA does interfere with the transcription termination activity of wild-type rho. The interference prevents interactions with RNA coupled to ATP hydrolysis.

Bear et al. (1985) have shown that limited tryptic digestion of the transcription termination factor rho yields predominantly two fragments

when the protein is bound to ATP and poly(rC). These results suggest that activated rho has two structural domains neither of which individually retains ATPase activity.

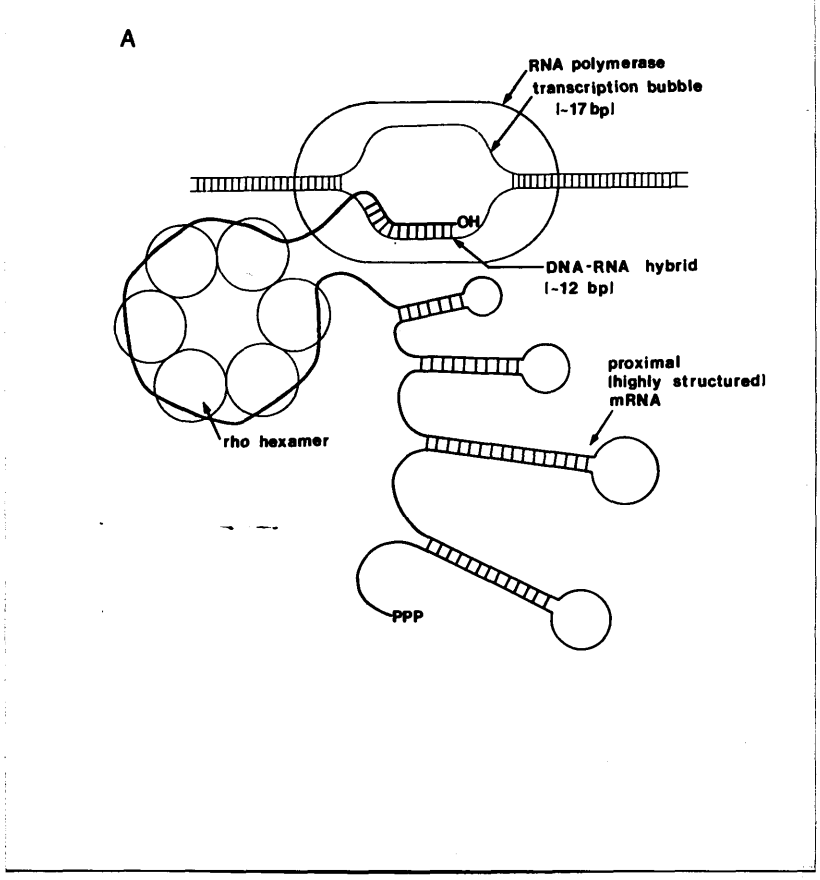
Rho releases nascent RNA chains from complexes in which elongation has been arrested. The release of the transcript is dependent on ATP hydrolysis. Experiments by Richardson and Conaway (1980) and Shigesada and Wu (1980) suggested that the hydrolysis produced a conformational change in the rho protein-ternary complex, resulting in dissociation of the nascent RNA chain.

The nucleic acid sequence of several different rho-dependent termination sites have been determined and no sequence homology has been observed. (von Hippel et al. 1984.) Several in vitro studies have shown that rho-dependent termination is not so precise but usually occurs at a series of sites which may be as far apart as 160 base pairs. (Kassavetis and Chamberlin 1981, Reisbig and Hearst 1981, Wu et al. 1981, Drahos and Szybalski 1981, Lau et al. 1982, Morgan et al. 1983a.) In the few studies where in vitro and in vivo termination sites have been compared (Court et al. 1980, Rossi et al. 1981) it has been shown that sites that give rise to rho-dependent termination in vivo correspond to the in vitro rho-dependent stop sites. Further work may not show this to be general.

Kinetic studies have shown that the multiple termination sites correlate with RNA polymerase pausing sites. (von Hippel et al. 1984.) These results indicate that rho-dependent termination sites are also RNA polymerase pause sites although not all pause sites are recognised by rho. (Kassavetis and Chamberlin 1981, Morgan et al 1983a&b.)

The currently accepted model for rho-dependent termination (Figure 2) has rho bind to a long stretch (72-84 base pairs in length) of cytidine-containing RNA that is largely free of secondary structure.

Figure 2:Model for rho-dependent termination.



The model suggests that paused RNA can be terminated only if a sufficiently large region of unstructured RNA is available on the nascent transcript to enable the rho protein to bind. Highly structured RNA prevents rho binding.

von Hippel et al. (1984).

Activation of the ATPase activity is required for the release of the paused RNA polymerase from the ternary complex.

2. Transcription/translation coupling and its importance in controlling levels of protein expression:

Transcription and translation processes occur in the same location in procaryotes. Once transcription has proceeded beyond the ribosome binding site it is theoretically possible for the ribosome to attach to this site and commence translation. Indeed, transcription/translation coupling does occur (Miller et al. 1970). Conditions which interfere with transcription/translation coupling will affect mRNA synthesis and therefore protein production. Studies of the attenuator regions of many of the amino acid operons in E.coli have shown that transcription and translation are coupled by RNA polymerase pausing and decoupled by ribosome pausing. Transcription/translation coupling has also been shown to be affected by codon usage.

(a) Attenuation:

The trp (Bertrand et al. 1975), his (Kasai 1974, Barnes 1978, DiNocera et al. 1978), phe (Zurawski et al. 1978), leu (Gemmill et al. 1979), ilvGEDA (Lawther and Hatfield 1980, Nargang et al. 1980), thr (Gardner 1982) and ilvB (Friden et al. 1982. Hauser and Hatfield 1983) operons of E.coli K12 are amino acid operons that are regulated by attenuation. According to the current model of attenuation, formation of alternative secondary structures in the transcript of the leader region controls transcription termination at a site immediately preceeding the structural genes of the operon. The degree of transcription termination is determined by the kinetics of translation of the transcript. The leader polypeptide is rich in the particular amino acid(s) for that operon and a shortage of the appropriately charged tRNA causes translating ribosomes to stall. This promotes the formation of a secondary structure in the RNA transcript called the antiterminator which prevents transcription termination and enables the polymerase to transcribe the

structural genes. When a ribosome translates the entire leader peptide without stalling it promotes the formation of an alternative secondary structure in the RNA transcript, the terminator, which then causes termination at the attenuator. The terminator site contains a region of GC-rich dyad symmetry and a run of consecutive A residues on the template DNA. The transcribed RNA can form a stem-loop structure followed by a series of U residues-characteristic of a rho-independent termination site. The attenuator model as described requires that the translation of the leader peptide coding region be closely coupled to transcription of the same region. (Yanofsky 1981.)

Much genetic and biochemical evidence in support of the model is avaliable. (Kolter and Yanofsky 1982). In the case of the trp operon, RNase T1 digestion patterns of in vitro synthesised leader RNA are consistent with the predicted secondary structures. Analysis of mutants has shown the importance of transcription/translation coulping. (Oxender et al. 1979, Fisher et al. 1985.) Evidence in support of the formation of the proposed secondary structures involved in the pausing and termination of transcription at the trp attenuator has come from the use of DNA oligomers (Fisher and Yanofsky 1984.). These oligomers were complementary to specific fragments of the trp leader sequence and they could bind to the appropriate regions preventing the nascent RNA from forming the proposed structures. The affects on termination of the oligomers binding at these sites was a measure of their involvement in transcriptional pausing.

(b) Affect of codon usage on transcriptional/translational coupling:

The genetic code shows redundancy for most amino acids with several different nucleotide triplets encoding the same species. (Crick 1966.) Whilst the genetic code is used universally by all organisms there are distinct codon preferences for highly expressed proteins. The codons that are predominantly used for the synthesis of major proteins differ from organism to organism.

(Ikemura 1981.) In E.coli most highly expressed genes use a subset of codons which are recognised by abundant tRNA species. (Grantham et al. 1980.) Grosjean and Friers (1982) found that those codons which are rarely used in E.coli are also accompanied by low levels of the recognising tRNAs. From this it was assumed translation of these rare codons would be limited by the availability of these minor tRNA species.

The first direct evidence that codon usage could affect protein synthesis was obtained from experiments by Robinson et al. (1984). They showed that replacing the commonly used E.coli arginine codons (CTG) for the rarely used AGG codons decreased the yield of protein product at high transcription rates.

Pedersen (1984) compared the rate of translation of mRNAs containing commonly used codons (for fus,tsf,tuf and rpsA genes) and two mRNAs rich in rare condons (for bla and lacI genes). The latter genes were present on the high copy number vector pBR322 containing the up-promoter mutation $lacI^{q}1$. In this strain the average times for translation were 50% slower than the rates calculated for the ribosomal proteins. The increase in time of translation was interpreted as being a result of an overall drain of the rare tRNAs cognate to the rare codons.

Bonekamp et al. (1984) have shown that the codon composition of the translated region upstream of the pyrE attenuator that preceeds the pyrE gene affects the frequency of mRNA chain termination. It also appears to affect the rate of polypeptide chain elongation. The translational efficiency of individual codons placed in an artificial leader polypeptide in front of the pyrE attenuator have been determined (Bonekamp et al. 1985). The results showed that replacing the three rare arginine codons (AGG) in the artificial leader peptide with the frequently used arginine codons (CTG) increased read through of the pyrE attenuator by the transcribing RNA polymerase. The frequency of termination at the wild type attenuator is modulated by changes

in the UTP pool. (Turnbough et al. 1983, Poulsen et al. 1984.) These changes alter the degree of saturation of the polymerase with UTP which alters transcription and translation coupling because of changes in the mRNA chain elongation rate. It appears that attenuation at this site is affected by factors such as codon composition that alter transcription/translation coupling.

3. mRNA stability:

Another potentially important element in the regulation of gene expression in procaryotes is the stability of messenger RNA. Messenger RNA stability has been shown to be influenced by post-transcriptional processing, retroregulators and, in some cases, by bacterial growth rates.

(a) Post-transcriptional processing:

The 3' end of the trp operon mRNA was found to terminate at a point 36 nucleotides beyond the last structural gene at a rho-independent site, trpt (Wu and Platt 1978). Deletions distal to this site resulted in readthrough in vivo (Guarente et al. 1979, Wu et al. 1980). Analysis of mRNAs produced in vitro revealed a second rho-dependent termination site, trpt' (Wu et al. 1980.) Although it was thought that the two terminators might interact it was shown that they functioned independently. The rho-dependent site was found to be much more effective at directing termination. (Mott et al. 1985.) The inability to detect mRNA species terminated at the rho-dependant site in vivo was postulated to be due to the rapid 3' exonucleolytic degradation of the RNA from the trpt' site to the trpt stem loop. Trpt is not an efficent termination site in vivo or in vitro and its major function may be to provide a secondary structure preventing mRNA degradation. This observation suggests an alternative function for all RNA stem loop structures formed during transcription. Their major function may be to protect the mRNA from exonucleolytic degradation rather than acting as termination sites.

In another form of post transcriptional processing messenger RNA may be protected by the formation of secondary structures as a result of other

components (eg proteins) binding to the RNA. This occurs for mRNAs for several E.coli ribosome proteins and the bacteriophage T4 gene 32 protein. (Lemaire et al. 1978, Fill et al. 1980, Nomura et al. 1980, Burton et al. 1983.) The mRNA for gene 32 protein is unusually stable. (Gorski et al. 1985.)

Processing by RNase III can alter the stability of mRNA. The bacteriophage lambda int gene protein mRNA when cleaved at the RNase III site is degraded faster than the unprocessed transcript. (Schmeissner et al. 1984.) Conversely, bacteriophage T7 mRNA's processed by RNase III are more stable. (Panayotatos and Truong 1985.) It is proposed that the mode of cleavage within the RNase III site determines the stability of the mRNA obtained. In those species where stability is enhanced a single cleavage leaves a folded structure at the 3' end of the mRNA and this stabilises the upstream message. Double cleavage at the site removes the folded structure and accelerates degradation.

Bacterial mRNAs are not usually processed by RNase III either because no such sites exist or they are inaccessible to the enzyme. However, bacterial ribosomal RNA and tRNA precursors are subject to RNase III processing.

(b) Retroregulators:

Regulation of a target gene by a cis-acting DNA sequence distal to it has been termed retroregulation. (Schindler and Echols 1981.) Experiments have shown that the cis-acting element sib negatively regulates the expression of the int gene located proximal to the sib element.

A positive retroregulator, the cry terminator, that controls the expression of the gene encoding the insecticidal parasporal crystal protein (cry) of Bacillus thuringiensis has also been identified. (Wong et al. 1983.) This sequence has been isolated and introduced into E.coli where it has been shown to enhance the expression of upstream genes (Wong and Chang 1986). The penicillinase (penP) gene was used to study the effects of the cry

terminator in E.coli. (Wong and Chang 1986.) The penP gene transcript normally terminates just beyond the coding sequence at a rho-independent termination site. The half-life of the penP mRNA was 2.0-2.5 minutes which is similar to most bacterial mRNA. When the penP terminator was replaced by the cry terminator the half-life of the mRNA was increased to 6 minutes. There was a concomittant increase in penicillinase activity. Based on the experimental data it was proposed that the stem-loop structure at the 3' end of the mRNA, which corresponds to the inverted repeat sequence in the cry retroregulator fragment, was responsible for the enhanced mRNA stability. However, terminators from the lpp gene of E.coli and the ery gene of Staphylococcus aureus which normally generate very stable mRNA species did not increase the stability of the penP mRNA. Therefore it appears that either the cry terminator or the penP gene-cry terminator combination is unique in its abilty to modulate gene expression. Secondary structure may not be the only mechanism that enhances mRNA stability in bacteria.

(c) Bacterial growth-rate:

Nilsson et al. (1984) investigated the effects of growth rates on the stabilities of 4 monocistronic E.coli mRNA species-ompA, lpp, cat and bla. The results obtained showed that the decay rates of the messages were affected differently by changes in the rate of cell growth. Both the very stable ompA mRNA and the cat mRNA showed similar growth-related affects. The half-lives decreased with decreasing growth-rate. In contrast, changes in growth rate had no affect on the stabilities of lpp and bla mRNAs. The effects of growth on mRNA stability do not appear to be related to the extent of mRNA secondary structure since both the ompA and lpp genes generate transcripts capable of extensive secondary structure formation but show different reactions to growth conditions. No adequate expla nation has been put forward to explain these results.

C. α Interferons and their use in determining DNA sequences which affect mRNA synthesis and stability in E.coli:

Interferons were used as a model system for studying the effects of changes in DNA sequence in gene expression.

1. Interferon:

Interferons are small inducible proteins with a wide range of species specific biological activities. They confer resistance to viral infection, activation of natural killer activity, inhibition of cell proliferation, enhancement of antibody-dependent cell-mediated cytotoxicity, and suppression of antigen and mitogen-induced leucocyte inhibition (Fellous et al. 1982 and Mizoguchi et al. 1985). There are three distinct serological types of human interferons termed α or leucocyte, β or fibroblast and γ or immune. The β and γ interferons are glycosylated species and their genes are present in single copies per genome. (Ohno and Taniguchi, 1981, Tavernier et al. 1981, Gray and Goeddel. 1982) In contrast the α interferons are non-glycosylated and are represented by a family of genes with a high degree of nucleic acid homology present in about 20 copies per genome. (Ngata et al. 1980a, Brack et al. 1981, Goeddel et al. 1981, Lawn et al. 1981 and Ullrich et al. 1982.) To date 13 nonallelic and 8 allelic α interferons have been identified (Mizoguchi et al. 1985).

The family of α interferon proteins, with a molecular weight range of 17,500 to 21,000, have been the subject of intense clinical interest. This, coupled with the inability to obtain large quantities of the purified proteins from natural sources, has promoted great interest in the production of α interferons by recombinant DNA procedures. Workers have expressed the α interferon sequences in various bacterial plasmid vectors under the control of different procaryotic promoters. (Nagata et al. (1980a&b), Goeddel et al. (1980), Strueli et al. (1980), Yelverton et al. (1981), Taniguchi et al. (1980), Remaut et al. (1981), Palva et al. (1983) and Slocombe et al. (1982).) Goeddel

et al. (1981) compared the sequences of eight distinct α interferons and found that they were approximately 80% homologous with respect to amino acid sequence-a total of 70 out of 166 (165 for α2 interferon). The overall distribution of amino acids along the molecule appeared to be random except between amino acids 115 and 151 where at 31 out of 37 postions the amino acid was invariant. It was suggested that the side-chains of some of these common residues contribute to structural elements critical for the shared biological properties (Camble et al. 1986). Despite this large degree of homology Streuli et al. (1980) showed that interferons α1 and α2 have striking differences in their antiviral activities on cells of different species.

2. Development of different α interferon constructs and their uses for studying the effects of changes in DNA sequences on mRNA and protein production:

Dr P.J. Cozens of Wellcome Foundation Ltd constructed several α1 (Figure 3) and α2 (Figure 4) interferon plasmids and cloned them into E.coli strain MM294. The α1 plasmids differed from each other with respect to copy number and the inclusion or omission of a transcription terminator, the threonine operon attenuator at the end of the 3' non-coding sequence. The α2 plasmids differed from each other in copy number, sequences 5' to the -35 region of the promoter, the length of the 3' non-coding region and in the distance between the "Shine-Dalgarno" region and the translation initiation codon. The plasmids in each group differed from each other by single DNA sequence changes to non-coding regions which resulted in altered protein production.

The aims of this investigation were to determine the levels of mRNA,the types of mRNA and the stabilities of the mRNA produced by all the constructions and to relate mRNA production to levels of interferon expression. This would determine the effects of specific DNA sequences on mRNA synthesis and stability. Comparison of identical α1 and α2 constructs

would determine whether changes in DNA coding sequence affect mRNA and protein production in E.coli.

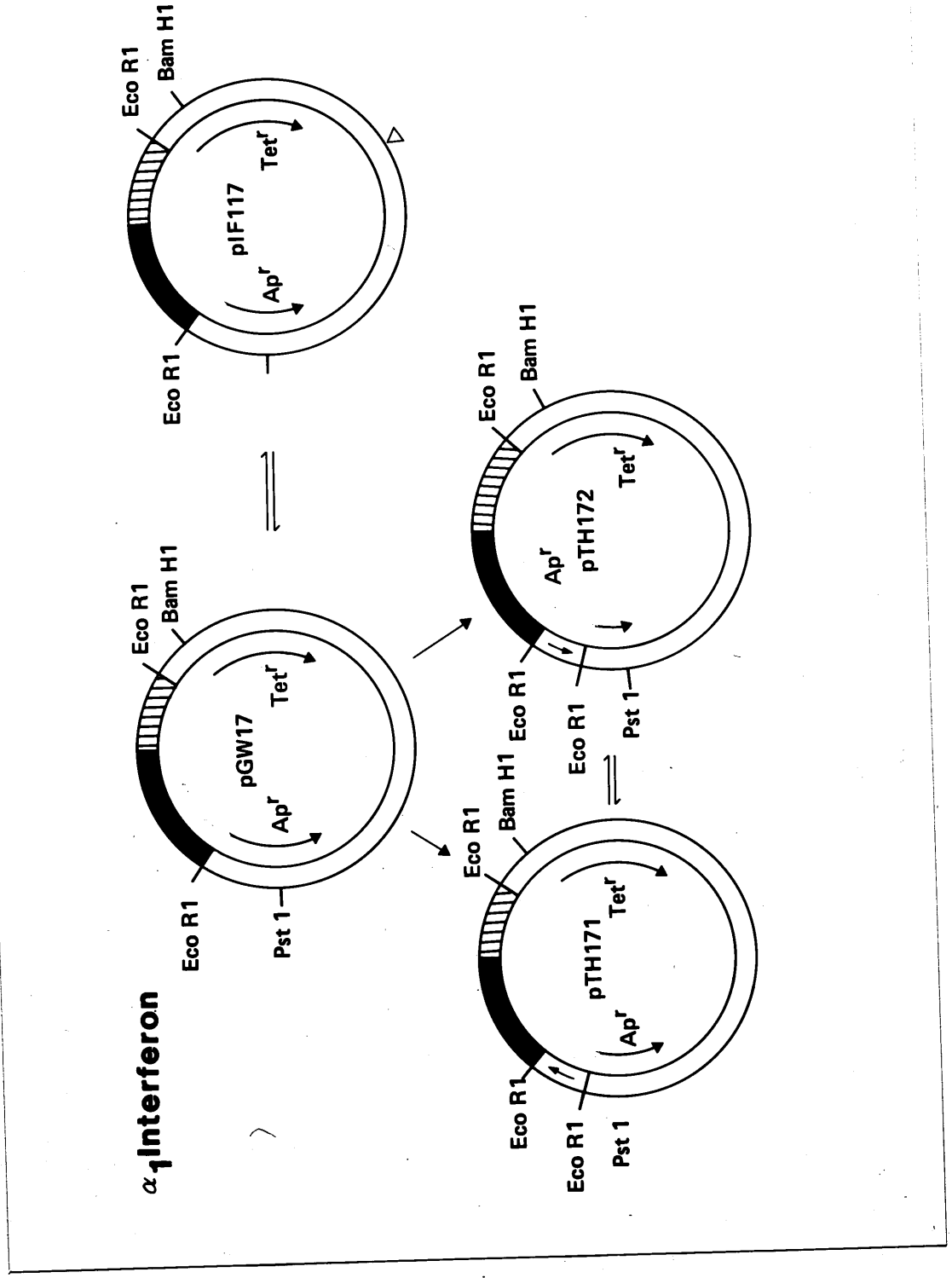
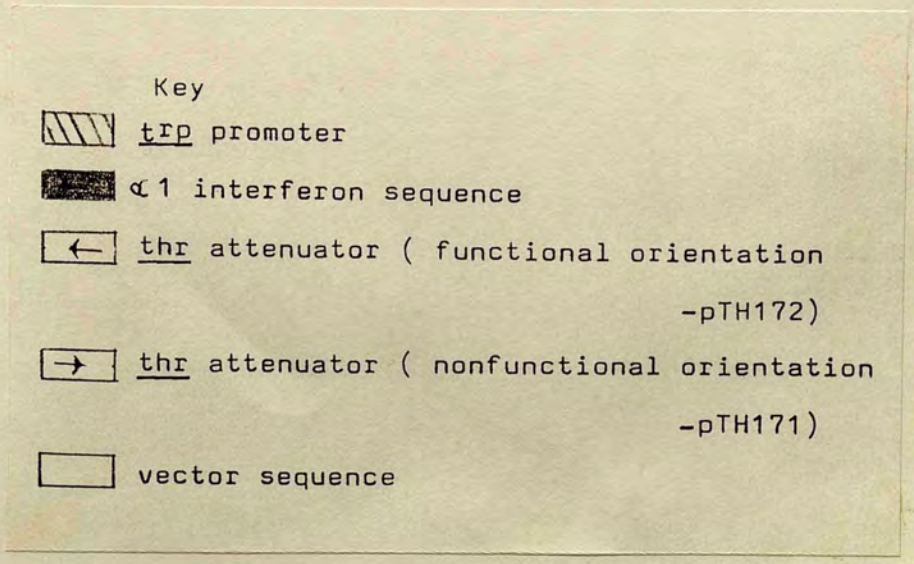
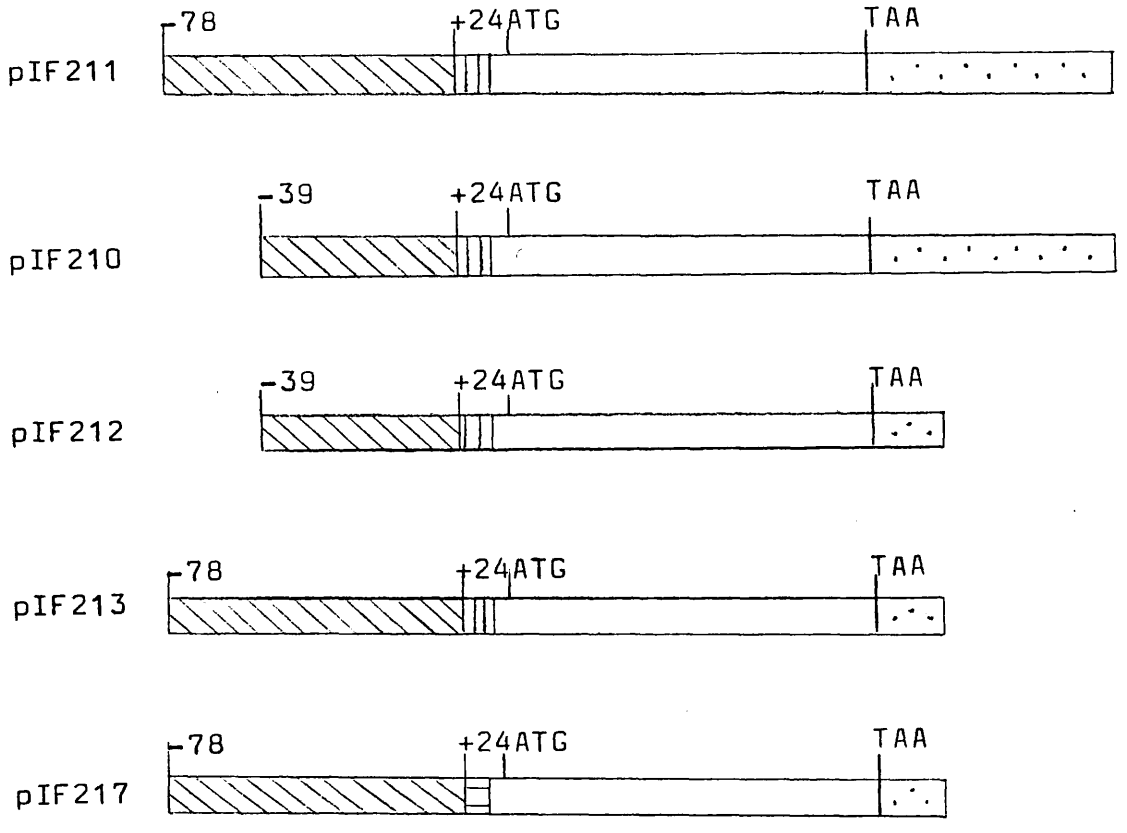

α1Interferon
Eco R1
Bam H1
pIF117
Ap^r
Tet^r
Eco R1
pGW17
Pst 1
pTH172
pTH171

Figure 3: Interferon α1 constructions.

Plasmid pGW17 was obtained from Cetus Inc. The unshaded portion represents the vector pBR322 cleaved at the EcoRI site. The hatched portion represents the tryptophan promoter of E.coli from nucleotides -78 to +24, using the coordinates of Bennett et al. (1978). This region contains the "-35" and "-10" regions of the trp promoter, the trp mRNA initiation site and the trp attenuator Shine-Dalgarno sequence. A CTTATG linker connects the trp sequence with the first codon of the mature interferon α1. The interferon sequence is represented in the diagram by solid shading. This plasmid contains some of the non-coding 3' region of interferon α1 cDNA up to nucleotide 635 (Goeddel et al. 1981). The EcoRI-flanked expression cassette was excised and inserted into the EcoRI site of the pBR322 deletion derivative pAT153 (Twigg and Sherratt 1980) to form pIF117. Plasmids pTH171 and pTH172 contain the threonine promoter and transcription terminator from the E.coli threonine operon attenuator inserted into an EcoRI site of pGW17 as shown in the figure. These are contained on a 367 base pair EcoRI fragment described by Lynn et al. (1982). The sequence of this fragment is shown in Gardner (1982). The arrows within this fragment show the direction of the transcription of this region when it resides on the E.coli chromosome.





Key

trp promoter

GAATTCATG linker

CTTATG linker

α2 interferon coding sequence

α2 interferon 3' non-coding sequence

Figure 4: Interferon α2 constructions.

The open lines denote vector sequences. Plasmid pIF217 contains the entire vector pBR322 cut at the EcoRI site. The triangles adjacent to the other plasmids denote that these contain the deletion derivative vector, pAT153 (Twigg and Sherratt 1980). The interferon α2 cDNA was cloned as described (Slocombe et al. 1982) and has the same sequence as interferon α2 of Goeddel et al. (1981). The stippled blocks represent interferon α2 cDNA from the first codon of mature interferon α2 to the 3' end of natural interferon α2 mRNA. The solid blocks represent the same cDNA terminating at nucleotide 616, using the coordinates of Goeddel et al. (1981). The hatched areas represent the tryptophan promoter of E.coli from nucleotides -78 to +24 in pIF211, pIF213, pIF215 and pIF217 and from nucleotides -39 to +24 in pIF210 and pIF212, using coordinates of Bennett et al. (1978). A GAATTCATG linker sequence connects the trp sequence with the first codon of mature interferon α2 in all plasmids except pIF215 and pIF217 where the linker sequence is CTTATG.

II: MATERIALS AND METHODS

Materials:

(α^{32}P) dATP (3000 Ci/mmol) and (α^{35}S) dATP (600Ci/mmol) were obtained from the Radiochemical Centre, Amersham.

Acrylamide. N, N'-bisacrylamide, urea, formamide, amberlite monobed resin MB-1, methylene blue, Deoxyribonuclease 1 and other Analar grade reagents were obtained from BDH Chemicals Ltd. Coomassie Brilliant blue, 2-mercaptoethanol, dithiothreitol, N, N, N', N'-Tetramethylethylenediamine (TEMED), ethanolamine, N' -Tris (hydroxymethyl) methyl glycine (trycine), Potassium morpholinopropane sulphate (MOPs), spermidine, salmon sperm DNA, IPTG, X-Gal, thiamine, tryptophan, threonine, methionine, isoleucine, valine, tyrosine, histidine, thymidine, agarose types I and II, ampicillin, tetracycline, rifampicin, lysozyme, polyvinylpyrrolidone, BSA, Triton X-100, isoamyl alcohol, ATP, dATP, dCTP, dTTP, dGTP, diethyl pyrocarbonate, yeast transfer RNA were supplied by Sigma (London) Chemical Company Ltd. Bacto tryptone, bacto casamino acids, bacto yeast extract and bacto agar were purchased from Difco Laboratories. Meat casein polypeptone (peptone 180) was supplied by Gibco Europe. Ficoll 40,000 mwt and Sephadex G-50 superfine came from Pharmacia Fine Chemicals. Pall Biodyne A was supplied by Fall Ultrafine Filtration Corp. Glen Cove, USA. M13 vectors, epoxy-activated cellulose, Nacs prepac columns, 1 kb ladder, 123 base pair DNA ladder and low molecular weight protein standards were supplied by Bethesda Research Laboratories (UK) Ltd. Klenow fragment of DNA polymerase I (or DNA polymerase I-large fragment) was supplied by NEN Research Products, Drieich, West Germany. Super AMV Reverse transcriptase was supplied by Anglian Bioteohnology Ltd. Restriction endonucleases AhaIII, BamHI, BglII, EcoRI, HindIII, PstI, PvuII, RsaI, TaqI and XhoII, polynucleotide kinase, calf intestinal alkaline phosphatase, DNA polymerase I, RNase, ddATP ddCTP, ddGTP, ddTTP and caesium chloride were supplied by Boehringer Mannhiem. Ribonucleoside-vanadyl complex and T4-DNA Ligase were supplied by New England Biolabs.

Interferon α1 plasmids pGW17, pIF117, pTH171 and pTH172 (Figure 3) and α2 plasmids pIF210, pIF211, pIF212, pIF215 and pIF217 (Figure 4) were constructed by Dr P.J Cozens, Wellcome Foundation Ltd.

Plasmids were transformed into E.coli strains MM294, DH1, HD152 and KH54 (Appendix A). (HD 152 and KH 54 were obtained from the E.coli Stock Centre. New Haven, USA.) M13 vectors were grown in E.coli strain JM103.

Methods:

A.General methods.

1.Growth of bacterial cultures:

Bacterial strains containing the interferon plasmids were stored in glycerol: L-broth (1:1) (Appendix B) at -20°C. Aliquots (75µl) were added to 10ml of liquid L-broth (Appendix B) containing ampicillin (40µg/ml) and grown overnight at 37°C in an orbital shaker. The overnight culture was diluted 100 fold into 10ml M9CA medium (Appendix B) containing ampicillin (40µg/l), tryptophan (40µg/l) and for strains pTH171 and pTH172, threonine (40µg/l) also. The bacteria were grown overnight at 37°C. 2.5ml of the M9CA overnight culture was added to 22.5ml of M9CA medium containing ampicillin (40µg/l) and tetracycline (12.5µg/µl) and for strains pTH171 and pTH172 threonine (40µg/ul) also. If interferon production was to be repressed tryptophan (40µg/l) was added. After 90 minutes incubation at 37°C 3β -indoleacrylic acid (IAA) (10mg/ml in ethanol) was added to induce interferon production in the appropriate cultures and incubation at 37°C was continued until the required absorbance 650nm was obtained. (A650=1.0, for mRNA extraction and plasmid copy number determinations. A650=1.5 for interferon bioassay and polyacrylamide gel analysis.)

2.Large scale plasmid preparations:

(Maniatis et al. 1982.) For each 250ml of final growth medium 5ml of liquid L-broth containing ampicillin (40µg/ml) was inoculated with 75µl of gycerol/L-broth culture and grown overnight at 37°C. The next day 2.5ml of this culture was inoculated into 12.5ml of liquid L-broth containing ampicillin (40µg/ml) and grown for 6-8 hours. An inoculum (10ml) from this culture was then added to 250ml of liquid L-broth containing ampicillin (40µg/ml), and 20% glucose (375µl) and left to grow at 37°C for 16 hours.

The bacteria were pelleted by centrifugation (GSA rotor, 10,000 rpm for 20 minutes). The bacterial pellet was resuspended in 8ml of sterile solution containing 50mM glucose, 25mM Tris.HCl, 10mM EDTA and 40mg of lysozyme. (The lysozyme was added to the solution immediately before use.) This was left to stand at room temperature for 5 minutes. Then 10ml of freshly prepared 0.2M NaOH containing 1% SDS was added, the contents mixed and incubated on ice for 10 minutes. After the addition of 12ml of an ice-cold solution of 5M potassium acetate (pH 4.8) the contents were mixed then incubated on ice for a further 10 minutes. The samples were centrifuged (JA20 rotor, 20,000 rpm for 20 minutes at $4^{o}C$) and to 9ml of the resulting supernatant 6ml of isopropanol was added. Samples were mixed and left to stand at room temperature for 15 minutes before centrifugation (JA20 rotor, 12,000 rpm for 30 minutes at room temperature). The pellet was redissolved in 8ml of TE buffer (10mM Tris.HCl, 1mM EDTA, pH8.0) and then recentrifuged (JA20 rotor, 10,000 rpm, 10 minutes). For every millilitre of the supernant 0.95g of solid caesium chloride and 1mg of ethidium bromide (10mg/ml) was added. Sealed samples were centrifuged in a Beckman ultracentrifuge (70 Ti rotor, 40,000 rpm, $20^{o}C$ for 65 hours).

Visualisation of the samples under UV (300nm) after centrifugation revealed two DNA bands and a pellet. The upper most band contained nicked circular DNA, the second band contained closed circular plasmid DNA and the pellet contained RNA. The closed circular plasmid DNA was collected and the ethidium bromide removed by addition of isopentyl alcohol.

Two alternative purification procedures yielding DNA of differing quality could be followed. For ultra-pure preparations such as those required for sequencing the plasmid DNA was dialysed at $4^{o}C$ in 2 litres of TE buffer with at least three changes of buffer. Alternatively, an equal volume of sterile distilled water was added to the samples followed by 2.5 volumes of absolute ethanol. The samples were placed in dry ice for 10 minutes, then

centrifuged (JA20 rotor, 10,000 rpm, 10 minutes). To this, 8ml of 70% ethanol was added, the samples vortexed for 2-3 minutes, centrifuged (JA20 rotor, 10,000 rpm, 10 minutes) and dried in a rotary evaporator. After resuspension in the required volume, the yield was calculated after measurements of DNA content at A 260nm. (A 260=1.0 corresponds to approximately 50µg/ml of double stranded DNA.)

3.Small scale plasmid preparations:

(Birboim and Doly 1979.) Small quantities of plasmids were prepared by alkaline lysis of the bacterial pellet. A modification to the procedure described in Maniatis et al. (1982) enabled larger quantities (4-8µg) to be prepared. Cultures (10ml L-broth and ampicillin (40µg/ml)) were grown to stationary phase overnight. Five times the stated amounts of solutions I, II and III were used in accordance with the protocol. However, after the the addition of solution 111 and subsequent centrifugation, the samples were ethanol precipitated at -20^{o}C for 30 minutes. DNA was pelleted by centrifugation (JA20 rotor, 12,000 rpm, 10 minutes, -10^{o}C) and resuspended in 180µl TE buffer and 20µl 3M sodium acetate (pH 5.8). Samples were purified in accordance with the published Maniatis procedure from this stage.

4.Southern transfer and hybridisation:

(Southern 1975.) DNA species were separated on a TBE (0.089M Tris-borate, 0.089M boric acid, 0.002M EDTA) 1% agarose gel. After electrophoresis the gel was placed in 150ml of denaturing solution (2.5M NaCl,0.5M NaOH) for 30 minutes then transfered to 150ml of neutralising solution (3M sodium acetate, pH5.5) for a further 30 minutes. Excess buffer was removed from the gel surface before Pall Biodyne A, presoaked in 10x SSC (1.5M NaCl, 0.15M sodium citrate pH7.0), was placed directly on top. Transfer was performed overnight in high salt buffer (3M NaCl. 0.3M sodium citrate. pH7.0) from the low saltgels. The filter was then dried at 80^{o}C for 1 hour.

In DNA dot blots the DNA was spotted directly onto the filter. After application the DNA was dried and dipped in denaturing solution for 30 seconds, then neutralising solution for 30 seconds. Excess buffer was removed and the filters baked at 80°C for 1 hour.

Filters were prehybridised in prehybridisation buffer. The buffer contained 1.0ml 10x SSPE (3.6M NaCl, 0.2M NaH_2PO_4/Na_2HPO_4, 20mM EDTA, pH7.7). 80µl 10% SDS, 0.2ml 100x Denharts solution (2% Ficoll (40,000 mwt), 2% polyvinylpyrrolidone (36,000 mwt), 2% BSA), 1.72ml water and 1.0ml denatured salmon sperm DNA (100µg/ml). (Salmon sperm DNA was denatured by incubating an alkaline solution containing the DNA at 65°C for 10 minutes then neutralising it with 0.1M HCl.) Filters were prehybridised for 1 hour at 65°C. Hydridisation buffer, identical to prehydridisation buffer but containing the radiolabelled probe denatured similarly to the salmon sperm DNA replaced the prehybridisation buffer and the filters were incubated at 65°C overnight. Filters were washed three times in 500ml of wash buffer (5mM Na_2HPO_4, 1mM EDTA, 0.2% SDS, pH7.0) at room temperature before being autoradiographed overnight.

5.RNA purification:

(a) Total RNA:

Cultures were grown as previously described (General Methods 1.) until they reached the required A 650nm. Samples (10ml) were removed and the cells chilled rapidly on ice. The bacteria were pelleted by centrifugation (JA20 rotor, 8,000 rpm, 10 minutes at room temperature) and lysed at room temperature in 0.15M sucrose/0.01M sodium acetate, pH4.5/1% SDS (von Gabain et al. 1983). An equal volume of hot phenol (60°C) was added and the solution thoroughly mixed (Scherrer and Darnell 1962). The samples were centrifuged (JA20 rotor, 15,000 rpm, 5 minutes at room temperature) and the supernatant transfered to a clean tube. This was extracted a further three times with phenol/chloroform/isoamyl alcohol (25:24:1) at room

temperature. Total RNA was precipitated overnight at -20°C with 2.5 volumes of ethanol and 0.1 volumes of 3M sodium acetate (pH5.5). After centrifugation (JA20 rotor, 12,000 rpm, 10 minutes at -10°C)the pellet was dried in a rotary evaporator then resuspended in 300µl of DPC treated water. The quality of the RNA was assessed by visualisation of ethidium bromide stained TBE 1% agarose gels containing 5µl of RNA sample per track. Undegraded RNA preparations contained two distinct ribosomal RNA bands (Figure 5). Contaminating DNA was also detected and could be removed by digestion with DNase I (free of RNase). DNase I (200 units per mg) was added and the sample was incubated at 25°C for 30 minutes. RNA was precipitated overnight at -20°C in 2.5 volumes of ethanol and 0.1 volumes of 3M sodium acetate (pH5.5). After centrifugation (JA20 rotor, 12,000 rpm, 10 minutes at -10°C) the pellet was dried in a rotary evaporator and resuspended in 300µl DPC treated water. RNA was quantitated spectrophotometrically (A260 of 1.0 corresponds to approximately 40µg/ml of RNA) and stored at -70°C.

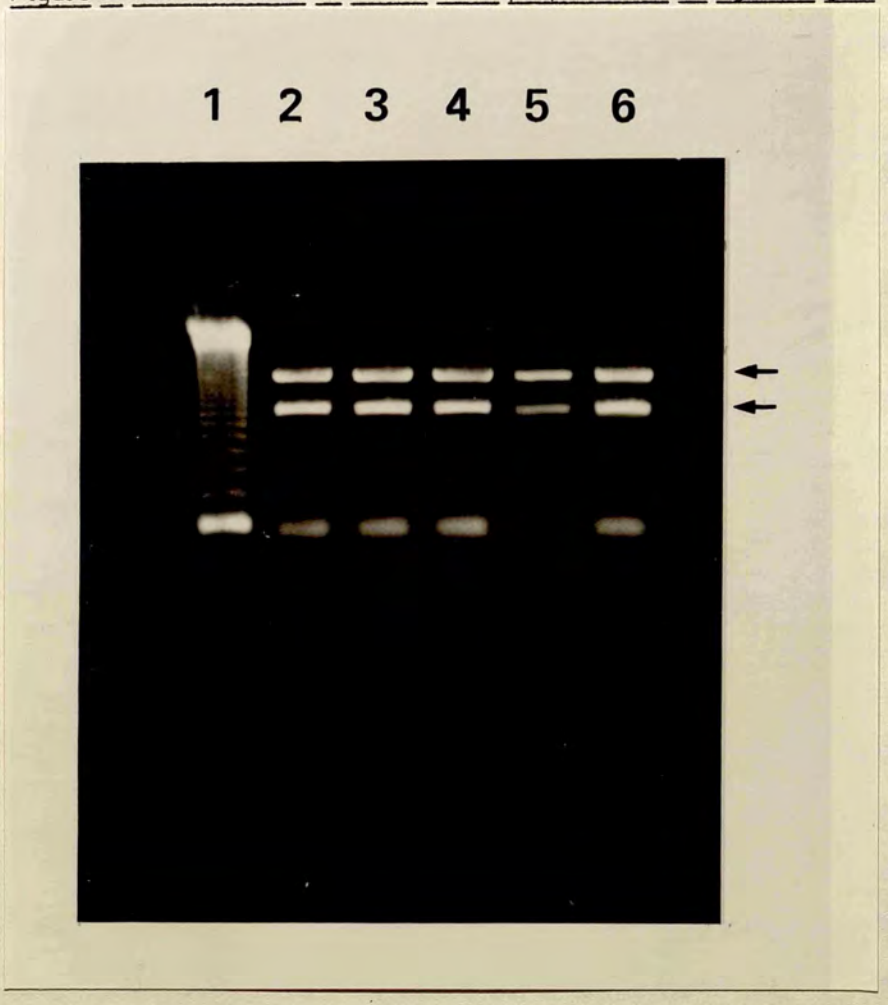
(b) RNA half-life determinations:

Starter cultures were grown as previously described but the final volume of growth media (M9CA) was increased to 75ml. Rifampicin (0.2mg/ml of a 50% ethanol solution) was added to cultures (A260 1.0) and samples (10ml) were removed at intervals of 0, 1, 2, 4 and 8 minutes after rifampicin addition. The samples were immediately placed on ice. Total RNA was extracted as described previously.

6. Northern transfers and hybridisation:

Glyoxal was mixed with Amberlite mixed bed resin (1g/5ml) before 5.8µl was added to 20µl of dimethyl sulphoxide, 4µl of 0.01M phosphate buffer pH7.0, 0.8µl of 10% SDS and 9.4µl of RNA (7.8 2µg). DNA standards (8µg of the 1 kilobase pair and 123 base pair DNA ladders) were similarly treated. The samples were incubated at 50°C for 1 hour, cooled to room temperature, 8µl of loading buffer (50% glycerol, 0.01M phosphate buffer pH7.0, 0.4%

Figure 5: Identification of intact RNA preparations on agarose gels.



Total cellular RNA (2µg) was isolated from construction pIF212 at 0, 1, 2, 4 and 8 minutes after rifampicin addition. The samples were separated by electrophoresis on a TBE 1% agarose gel containing ethidium bromide (General methods 8) and visualised under U.V. 300nm.

Lane 1, 123 base pair DNA ladder. Lane 2, 0 minutes. Lane 3, 1 minute. Lane 4, 2 minutes. Lane 5, 4 minutes. Lane 6,8 minutes.

The arrows indicate the prominent ribosomal RNA bands present in all the tracks.

bromophenol blue) was added and samples were loaded onto a 1% agarose gel. The gel was poured and run in 0.01M phosphate (pH7.0) at 120 volts for 3-4 hours. The buffer was constantly recirculated. Upon completion of electrophoresis the RNA was transfered to Pall Biodyne A by the method of Thomas (1980). Transfer was performed in high salt (20 x SSC:3M NaCl, 0.3M sodium citrate, pH7.0) from low salt phosphate (0.01m) gels. The Biodyne A membrane was prewet with 10 x SSC before contact with the gel. Transfer of the RNA from the gel to the membrane was allowed to proceed for at least 16 hours at room temperature after which time the membrane was baked under vaccuum at 80^{o}C for 45 minutes. The section containing the DNA size markers was removed and soaked in 5% acetic acid for 15 minutes. It was then transfered to a solution of 0.5M sodium acetate (pH 5.2) and 0.04% methylne blue for 5-10 minutes. The membrane was rinsed in water for 5 minutes to develop the bands. The remainder of the membrane containing the RNA could be used immediately or stored at 4^{o}C for up to two weeks.

Prehybridisation solution containing 7.5ml of formamide (deionised using a mixed bed ion exchange resin), 750µl of 100 x Denhart's (1966) (2% (w/v) ficoll 40,000 mwt, 2% polyvinylpyrrolidone 36,000 mwt, 2% BSA), 3.75 ml 20 x SSPE (3.6M NaCl, 0.2M sodium phosphate pH8.3, 0.02M EDTA), 450µl 10% SDS, 375µl of denatured salmon sperm DNA and 2.275ml of water was incubated at 100^{o}C for 5 minutes before addition to the membrane. The membrane was then incubated at 37^{o}C for 4-6 hours in the prehybridisation solution. For hybridisation overnight at 37^{o}C the prehybridisation solution was replaced with similarly prepared hybridisation solution containing the radiolabelled probe.

After hybridisation the membrane was washed three times in 400ml 2 x SSC + 0.1% SDS at room temperature, followed by two washes at 50^{o}C in 0.1 x SSC + 0.1% SDS. The membrane was then autoradiographed for as long as required.

RNA samples (8µg) could be dotted directly onto Pall Biodyne A, dried under vaccuum at 80°C for 45 minutes and then prehybridised. These dot blots were prehybridised in the previously described solution for 1 hour at 65°C then hybridised overnight at 37°C in hybridisation solution. The filters were washed 3 times with 2 x SSC + 0.1% SDS at room temperature and twice with 0.2 x SSC + 0.1% SDS at 50°C and then autoradiographed for 3 hours.

7. Nick translation:

Radioactive $[\alpha^{32}P]$ dATP was incorporated into DNA fragments by nick translation, a modification of the method of Rigby et al. (1977). DNA (1µg), 3µl 1M Tris.HCl pH8.0, 0.25M $MgCl_2$, 5µl BSA (1mg/ml), 2µl 0.48M 2-mercaptoethanol, 2.5µl 4mM dTTP, 2.5µl 4mM dCTP, 2.5ul 4mM dGTP and water, to make a final volume of 82.5µl were mixed on ice. To this 5µl of pancreatic DNase I (0.1µg/ml) was added and the solution incubated at 25°C for 1 minute and then returned to ice. Radioactive dATP, 5ul $[\alpha^{32}P]$ dATP (125µCi), and E.coli DNA polymerase I (40 units) were added and the sample was incubated at 15°C for 2 hours. Carrier, yeast transfer RNA (100µg), was then added and the labelled DNA separated from the unreacted nucleotides by chromatography on a Sephadex G-50 superfine column (150mm x8mm). The column was equilibrated and run in TE buffer and the separation of the two radioactive bands monitored by a hand monitor. The labelled DNA eluted immediately after the void volume and was precipitated overnight at -20°C in 2 volumes of ethanol and 0.1 volumes of 3M sodium acetate (pH5.5). The sample was centrifuged, (JA20 rotor, 12,000 rpm, 10 minutes at -10°C) dried in a rotary evaporator, redissolved in 100µl of water and the amount of radiolabel incorporated determined. Samples contained at least 1×10^7 dpm/µg (Cerenkov counts).

8. Agarose gel electrophoresis:

(Sharp et al. 1975.) Horizontal gels, 1% agarose, were poured and run in either TBE or TAE (0.04M Tris-acetate, 0.002M EDTA) buffer. Samples

contained loading buffer (TBE or TAE, 2% ficoll, 0.02% bromophenol blue). Ethidium bromide (0.5mg/l) was added to the running buffer and gels were run overnight at 30 volts. Baby gels were run at 100 volts for one hour.

9. Polyacrylamide gel electrophoresis:

Polyacrylamide gel electrophoresis was used for two purposes. Firstly, to separate and identify small DNA fragments and secondly, to separate proteins. For the separation of small DNA fragments non denaturing gels were used. These were 8% acrylamide poured and run in TBE buffer. Gels were run overnight at 40 volts and stained in ethidium bromide (0.5mg/l) for 20 minutes after electrophoresis had been completed.

Total cell proteins were separated by the method of Laemmli (1970). The equivalent of 1ml at $A650_{nm}$ 1.0 (approximately 200ug of protein) was centrifuged in an eppendorf bench centrifuge for 5 minutes and the sample resuspended in 50µl of water. To this was added 48µl of 2 x Laemmli buffer and 5µl of 2-mercaptoethanol. The sample was mixed, heated at $100^{o}C$ for 5 minutes and then 10µl of the sample loaded per track. The SDS containing gel was run in Tris.glycine buffer pH8.3 containing 1% SDS at 40 volts overnight or 35mA for 2-3 hours. Proteins were stained with coomassie blue.

10. Electroelution:

(Mc Donnell et al. 1977.) DNA fragments separated by agarose or polyacrylamide electrophoresis could be recovered from the gel by eleotroelution. For small fragments, <1 kilobase electroelution from polyacrylamide gels was unnecessary as the DNA diffused out of the gel. The rate of diffus ion could be increased if the gel was cut finely and then resuspended in 5ml of EE buffer (5mM Tris, 2.5mM acetic acid). Larger fragments were cut out of the gel, chopped finely and then placed into dialysis membranes with approximately 5ml of EE buffer. Membranes were submerged in the electroelution buffer and subjected to 200 volts for 2 hours. The polarity of the current was reversed for 10 minutes to remove any DNA

attached to the walls of the dialysis membranes. Samples were concentrated on Nacs prepac columns as per the manufacturers instructions. Samples were loaded onto the columns in low salt buffers, eluted in high salt buffers and then precipitated overnight at -20°C in 2 x volumes of ethanol. After centrifugation, (JA rotor, 12,000 rpm, 10 minutes at -10°C) the samples were dried in a rotary evaporator, resuspended in 25µl of water and stored at -70°C.

Purity and quantity of the samples were determined by agarose gel electrophoresis and visualisation of the ethidium stained gel under UV 300nm.

11. Transformation:

Competent cells were prepared and transformed by the method of Mandel and Higa (1970). Into 10ml of L-broth 75µl of glycerol/L-broth culture was inoculated and the bacteria were grown overnight at 37°C (30°C for strain HD152). The overnight culture was diluted into L-broth (1:100) and grown at 37°C (30°C for strain HD152) until A650nm=0.45-0.55. The cells were placed on ice for 15 minutes then harvested by centrifugation (JA20 rotor, 8,000 rpm, 10 minutes at 4°C). As much medium as possible was removed from the pellet before it was resuspended in half the original volume of ice-cold 0.1M $MgCl_2$. The cells were centrifuged (JA20 rotor, 8,000 rpm, 10 minutes at 4°C) and the pellet resuspended in 1/20 the original volume of ice-cold 0.1M $CaCl_2$. The cells were then left on ice for at least an hour before use. Any unused competent cells were aliquoted (0.2ml) into tubes, frozen in an ethanol-dry ice bath and stored at -70°C. Competent cells were prepared from E.coli strains MM 294, DH 1, JM103, KH 54 and HD 152.

Plasmid DNA (20ng) was added to competent cells (0.2ml) and incubated on ice for 30 minutes. (Frozen competent cells were thawed slowly on ice.) The samples were transfered to preheated tubes and incubated at 42°C for 2 minutes. L-broth (0.5ml) was added and the samples then incubated at 37°C to enable the expression of the antibiotic selection marker. (HD152 was

incubated at 30°C.) A fraction (100ul) of the total sample was spread on to L-broth plates containing the appropriate antibiotics and the plates incubated at 37°C (HD152 30°C) overnight. The remainder of the sample was stored at 4°C. Samples were stored for only 24 hours.

12. Preparation of interferon for assay:

Cultures were grown as described in General Methods 1 until A650nm=1.5. The cells were then placed on ice for 10 minutes and the absorbance at 450nm measured. Cells were pelleted by centrifugation (JA20 rotor, 8,000 rpm, 10 minutes at room temperature), resupended in 1ml of 50mM Tris.HCl pH8.0, 30mM NaCl and transfered to Eppendorf tubes. Lysozyme (250µl, 10mg/ml in 30mM Tris.HCl pH8.0, 30mM NaCl) was added and the samples incubated on ice for 30 minutes. They were then freeze thawed 4 times using an ethanol/dry ice bath and a 37°C water bath. (The samples could be stored at this stage at -20°C.)

After freeze-thawing the samples were centrifuged (JA20 rotor, 10,000 rpm, for 30 minutes at 4°C) then transfered to thick-walled polycarbonate tubes before recentrifugation (50Ti rotor, 40,000 rpm, $w^2t=9.04 \times 10^9$ at 20°C). The resulting supernatant (S 100 lysate) was collected and assayed by the method of Rhodes et al. (1986). The samples could be stored at -70°C.

Samples (50µl) were added to 45µl 2x Laemmli buffer and 5µl 2-mercaptoethanol, incubated in a boiling water bath for 5 minutes, cooled then loaded onto SDS polyacrylamide (15%) gels and electrophoresed as described (General Methods 9). The gels were soaked in 10% TCA for 15 minutes before coomassie blue staining in order to minimise loss of interferon.

B. Methods:

1. Plasmid copy number:

Plasmid copy numbers were determined by the method of Nugent et al. (1983). Bacterial cultures were grown as described in General Methods 1.

Cells were pelleted by centrifugation (JA20 rotor, 8,000 rpm, 10 minutes at room temperature) then resuspended in water at a concentration of A650=20 units/ml. To 100µl of bacterial suspension, 100µl of 2 x TBE buffer and 50µl of lysis loading buffer (10% Ficoll, 5% SDS and 0.1% bromophenol blue) were added and the samples heated at 65°C for 30 minutes. RNase (5µl of a 1mg/ml solution) was added and the samples vortexed for 30 seconds. Samples (50µl) were loaded onto a 1% agarose gel poured and run in TBE buffer. The gel was run overnight at 40 volts in buffer containing ethidium bromide (0.5ug/ml). A photograph of the gel taken in UV (300nm) with a positive/negative film enabled quantitation of the plasmid DNA. The negative was scanned with a densitometer and the amount of plasmid DNA determined from the area under the graph of the densitometer tracing. Plasmid copy numbers were expressed relative to pBR322. The number of copies of pBR322 present per chromosome equivalent (20) was determined from the following equation:

$$\text{plasmid copies} = \frac{\text{amount of plasmid DNA} \times 3.8 \times 10^3 \text{ kb}}{\text{amount of chromosomal DNA} \times \text{plasmid size (kb)}}$$

2. Plasmid stability studies:

Bacterial cultures of E.coli strain MM294 containing the α1 and α2 plasmids and pBR322 and pAT153 were grown in liquid L-broth for 40 generations. Samples of each plasmid containing culture were then plated out on L-broth plates containing; (i) no antibiotics, (ii)ampicillin (40µg/ml) only (pIF210 and pIF212) or ampicillin (40µg/ml) plus tetracycline (12.5µg/ml) (the remainder). Those plasmids which were unstable were readily detected as the number of colonies present on the plates containing antibiotics were less than those on the control plates containing no antibiotics.

3. Preparation of hybridisation probes:

(a) Interferon α1 sequence specific probes:

(i)676 base pair fragment:

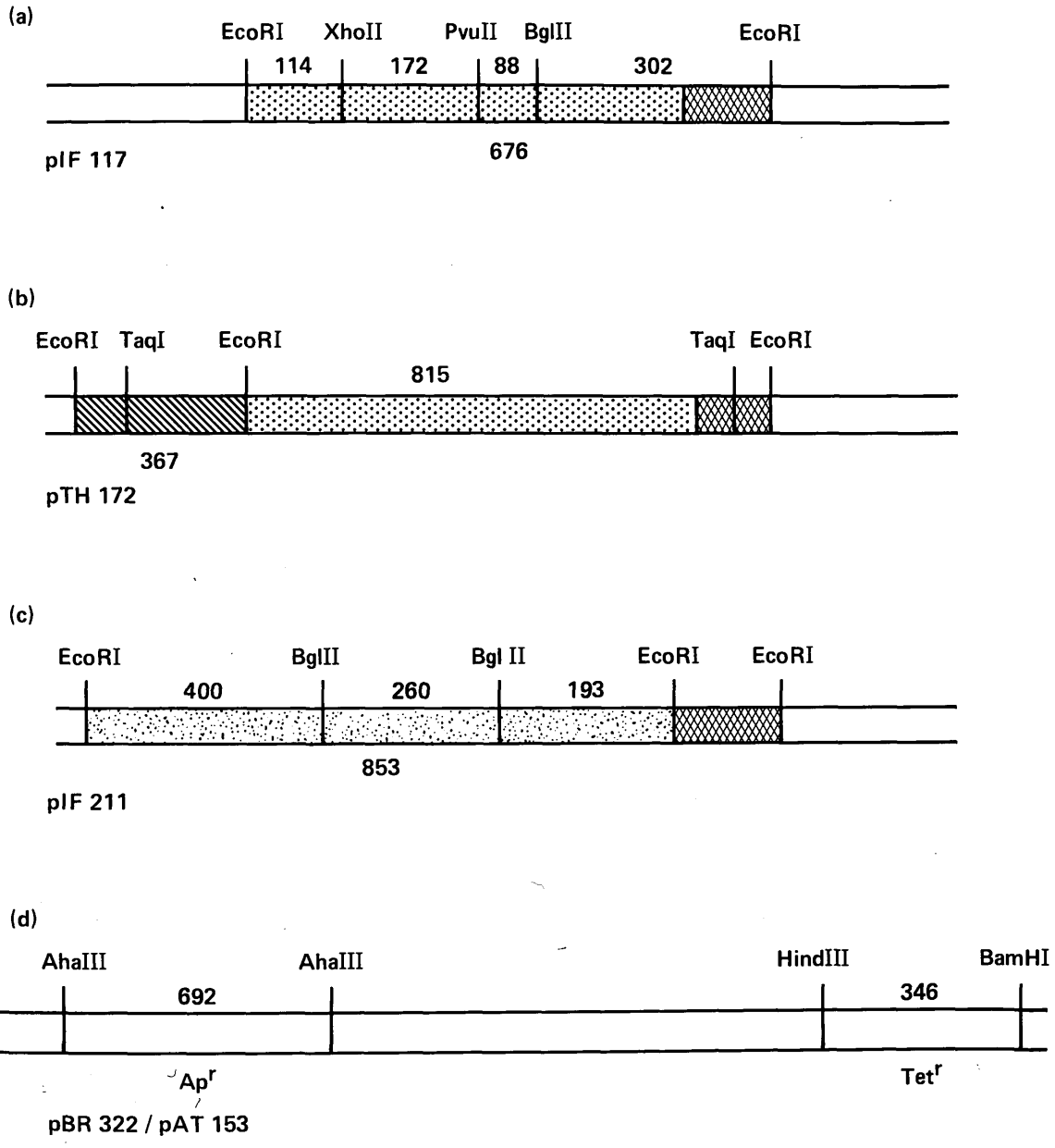
A fragment containing the entire α1 interferon sequence was prepared by EcoRI digestion of pIF117 (Figure 6 (a)). Purified pIF117 (180µg) and EcoRI (500 units) were incubated at 37°C in EcoRI incubation buffer (100mM Tris.HCl, 50mM NaCl, 10mM $MgCl_2$, pH7.5) for 3 hours. Digestion yielded two DNA species, one 676 base pairs in length containing the entire α1 interferon sequence, the other, 3655 base pairs in length. The two species were separated on a TAE 1% agarose gel. The required fragment was cut out of the gel and reset into a TAE 1.2% agarose gel. The fragment was electrophoresed on a total of 4 TAE agarose gels ranging from 1-1.5% agarose. After removal from the final TAE agarose gel the DNA was electroeluted then concentrated by passage through a Nacs prepac column. The fragment was redissolved in 25µl of water.

Experiments had shown that samples that appeared homogeneous after separation on a single agarose gel did contain contaminating DNA. Nick translated EcoRI digested pIF117 was used to probe Southern blots containing pIF117 and pBR322 DNA. The fragment hybridised to both DNAspecies (Figure 7). Electrophoresis on a second TAE agarose gel revealed that the higher molecular weight species was still present. Purification by electrophoresis through 4 consecutive agarose gels produced a fragment which when nick translated hybridised to only pIF117 DNA on Southern blots.

An alternative system for producing hybridisation probes using M 13 vectors was assessed. Plasmid pIF117 was digested with EcoRI and the resulting fragments separated on a TAE 1% agarose gel. The 676 base pair fragment containing the α1 interferon sequence was electroeluted and concentrated by passage through a Nacs prepac column. M13 vector, mp18 (1µg) was digested with EcoRI. (M13mp18 (1µg),4µl 5 x EcoRI incubation

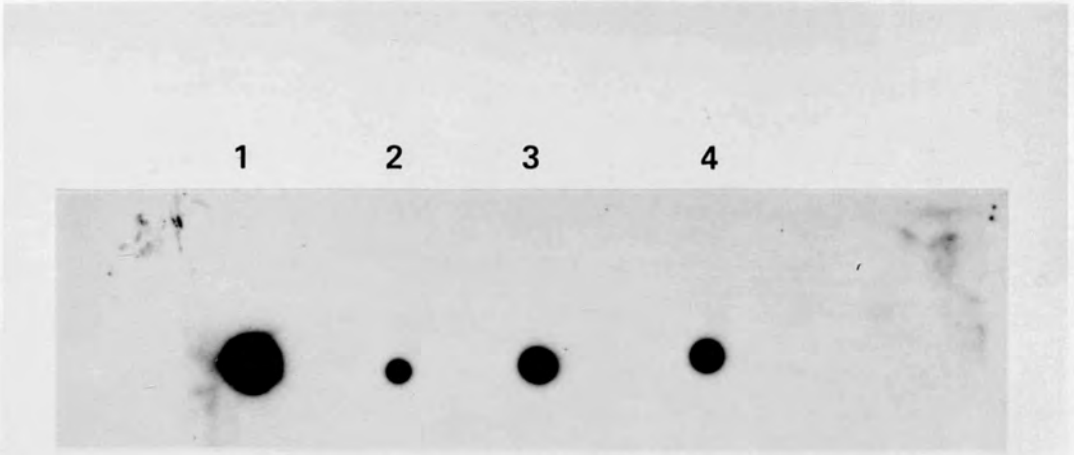
Figure 6: Diagrammatic representation of the DNA fragments obtained from Interferons α1 and α2 and the vectors pBR322 and pAT153 and used as hybridisation probes.

The DNA fragments obtained from vector and interferon sequences and used as hybridisation probes are shown in (a), (b), (c) and (d). Diagram (a) shows the interferon α1 specific DNA probes. DNA fragments containing the threonine operon attenuator region (367 base pairs) or most of the α1 sequence in addition to the threonine operon attenuator region (815 base pairs) are shown in diagram (b). Interferon α2 specific DNA fragments are shown in diagram (c) Vector based DNA fragments are shown in diagram (d).


(a)
EcoRI
XhoII
PvuII
BglII
EcoRI
114
172
88
302
676
pIF 117
(b)
EcoRI
TaqI
EcoRI
TaqI
EcoRI
815
367
pTH 172
(c)
EcoRI
BglII
Bgl II
EcoRI
EcoRI
400
260
193
853
pIF 211
(d)
AhaIII
AhaIII
HindIII
BamHI
692
346
Ap^r
Tet^r
pBR 322 / pAT 153
Key
trp promoter
α1 interferon sequence
thr attenuator
α2 interferon sequence
vector sequence

33054-8

Figure 7: Analysis of the specificity of hybridisation of DNA fragments purified by single agarose gel electrophoresis.



DNA (1µg) was applied directly onto filters and probed with a nick translated 676 base pair interferon α1 DNA fragment prepared as described in Methods 3, (a), (i).

Lane 1, pIF117. Lane 2, pBR322. Lane 3, pIF211. Lane 4, pAT153.

buffer (500mM Tris.HCL, 250mM NaCl, 50mM $MgCl_2$, pH7.5), 0.5µl EcoRI (10 units/µl) and water, total volume 20µl were incubated at 37°C for 2 hours. Calf intestinal alkaline phosphatase (1µl containing 25 units) was then added and the sample incubated at 37°C for a further 1 hour. Phenol extraction was followed by chloroform extraction before ethanol precipitation in 2.5 volumes of ethanol and 0.1 volumes of 3M sodium acetate (pH5.5). After centrifugation (eppendorf bench centrifuge for 5 minutes) the sample was dried in a rotary evaporator and redissolved in 50µl of water (20ng/µl).

Ligation reactions were performed in a total volume of 10µl. Vector (20ng), pIF117 676 base pair fragment (6ng), 1µl 10x ligase buffer (600mM Tris.HCl, 10mM EDTA, 100mM $MgCl_2$, pH7.2), 1µl 1mM ATP and 0.8µl T4 DNA ligase (Biolab, 400units/µl) were incubated overnight at 4°C. The ligation mix was then transformed into competent JM 103 cells and transformants, white plaques, were selected after overnight growth at 37°C on plates containing 40µl 100mM IPTG and 40µl X-gal, 2% in formamide. Appropriate controls containing cut vector, cut and religated vector (ligation control) and uncut vector were also included.

Templates were prepared from the selected white plaques. Each plaque was picked and grown in 2ml of 2 x TY medium (Appendix B) and 20µl of overnight JM 103 for 5-6 hours at 37°C with vigorous shaking. The samples were centrifuged for 8 minutes in an Eppendorf centrifuge and 1.2ml of supernatant was removed and added to 300µl of 20% PEG + 2.5M NaCl. The samples were mixed gently and left at room temperature for 15 minutes. After centrifugation (eppendorf bench centrifuge) for 5 minutes the supernatant was discarded and the samples centrifuged for a further minute. They were resuspended in 200µl TE buffer, extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), then with chloroform and finally 400µl of ethanol was added and the samples were precipitated overnight at

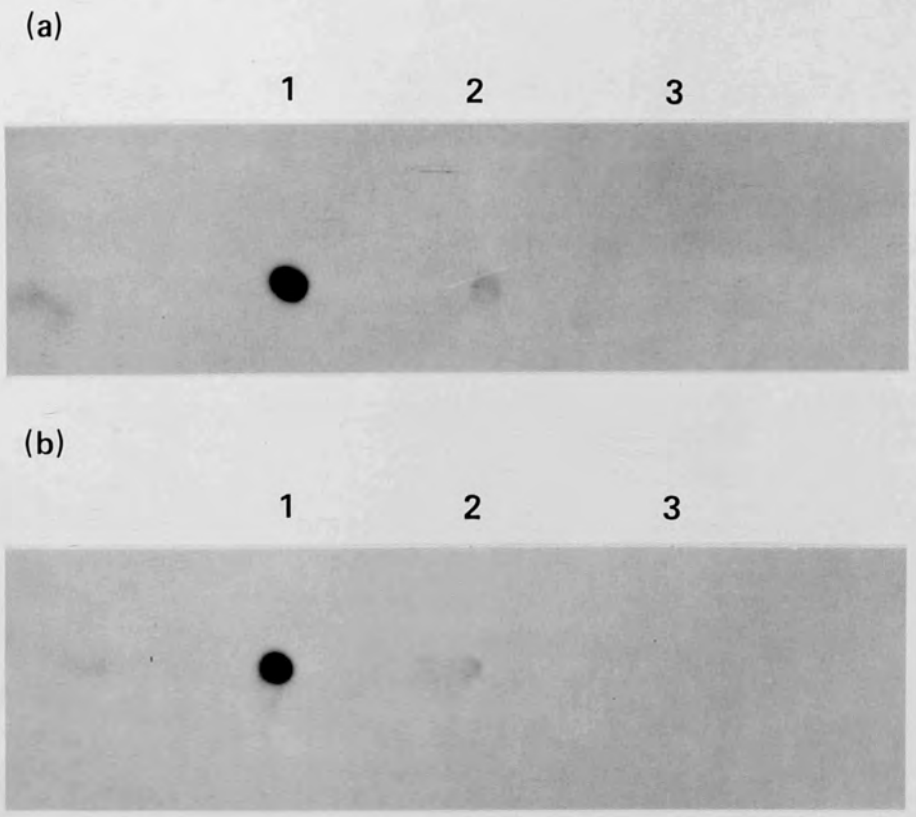
-20°C. After centrifugation (JA rotor, 12,000rmp, 10 minutes) the samples were dried in a rotary evaporator and resuspended in 10µl of TE buffer and stored at -20°C.

The inserted sequence was rendered partially double stranded by primed synthesis using a oligonucleotide primer complementary to a region 5' to the cloning site. (Method of Hu and Messing (1982).) Primer (2µl), template (50µg), water (3µl),0.1M dithiothreitol and 1µl 10 x HincII buffer (100mM Tris.HCl, pH7.0, 600mM NaCl, 66mM $MgCl_2$) were boiled together for 2.5 minutes then cooled slowly to room temperature. Klenow fragment of E.coli DNA polymerase I (5 units), 500µM dCTP, dTTP, dGTP (1µl) and $[\alpha^{32}p]$ dATP (25 µCi) were added and the reaction allowed to proceed at room temperature for 90 minutes. The unreacted nucleotides were separated from the labelled DNA by passage through a 150mm x 8mm G-50 superfine Sephadex column. The sample was resuspended in 100µl of water after centrifugation (JA20 rotor, 12,000 rpm, 10 minutes at 10°C) and drying of the resultant pellet. The probe (1×10^8 dpm/µg) hybridised only to the appropriate sequences on Northern and Southern dot blots (Figure 8). Although probes of higher specific activities were obtained by this method, preparation of DNA fragments by consecutive gel electrophoresis, electroelution and concentration by chromatography on Nacs prepac columns was preferred.

(ii) 367 base pair fragment:

A 367 base pair fragment containing the entire E.coli threonine operon attenuator region was purified from pTH172 (Figure 6 (b)). pTH172 (50µl, 65µg), 4µl of EcoRI (320 units), 20µl of EcoRI incubation buffer and 26µl of water were incubated at 37°C for 2 hours. EcoRI digestion of pTH172 yielded 3 fragments-367, 675 and approximately 4,300 base pairs in length. These fragments were separated on a TAE 1% agarose gel and purified by further TAE agarose gel electrophoresis, electroelution and chromatography on Nacs prepac columns. The purified samples were resuspended in 25µl of water.

Figure 8: Analysis of the specificity of hybridisation probes produced in M13 vectors.



Samples of DNA (1µg) and RNA (8µg) were applied directly onto filters and hybridised as described in General methods 4 (DNA) and 8 (RNA) to single-stranded probes prepared from M13 vectors containing the 676 base pair α1 interferon sequence.

Results of DNA hybridisation are shown in (a) and the results of RNA hybridisation are shown in (b).

Panel (a): Lane 1, pIF117. Lane 2, pIF211. Lane 3, pBR322.

Panel (b): Lane 1, pIF117. Lane 2, pIF211. Lane 3, pBR322.

(iii) 302, 88, 172 and 114 base pair fragments:

Fragments 302, 88, 172 and 114 base pairs long were obtained by digestion of pIF117 with enzymes EcoRI, BglII, PvuII and XhoII. The fragments spanned the entire α1 interferon sequence (Figure 6(a)). pIF117 (100μg), 7.5μl of 140mM 2-mercaptoethanol, 7.5μl of BSA (2mg/ml), 3μl EcoRI (80 units/μl), 10μl BglII (2 units/μl), 2μl PvuII (20 units/μl), 60μl water and 30μl of 5 x PvuII buffer (30mM Tris.HCl, 300mM NaCl, 30mM $MgCl_2$, 0.05% Triton X-100 (v/v), pH7.4) were incubated at 37°C for 4 hours. Three bands were visible under UV after TAE 1% agarose gel electrophoresis. The fragments 88, 286/302 and 3656 base pairs long were purified by agarose gel electrophoresis, electroelution and chromatography on Nacs prepac columns. The sample containing the 286 and 302 base pair fragments, which co-purified,was digested with XhoII. Three DNA fragments 302, 172 and 114 base pairs were obtained after purification of the XhoII digest. (Sample (25μl), 5μl 140mM 2-mercaptoethanol, 20μl of 5 x XhoII incubation buffer (30mM Tris.HCl, 30mM $MgCl_2$, 0/05% (v/v) Triton X-100, pH7.5), 6μl XhoII (1.8 units/μl) and 44μl water were incubated at 37°C for 3 hours.)

(iv) 815 base pair fragment:

Plasmid, pTH172 (140.5μg), 60μl 5 x TaqI incubation buffer (50mM Tris.HCl, 500mM NaCl, 25mM $MgCl_2$, pH8.0), 15μl 140mM 2-mercaptoethanol, 15μl TaqI (3.5units/μl) and 95μl of water were incubated at 65°C for 3.5 hours. Digestion of pTH172 by TaqI produced 9 DNA fragments (42, 88, 313, 315, 474, 616, 815, 1307 and 1444 base pairs in length). The 815 base pair fragment contained most of the α1 interferon sequence and 246 base pairs of the threonine operon attenuator (Figure 6(b)). The fragments were separated by TAE 1% agarose gel electrophoresis and the 815 base pair fragment reset into TAE 1.2% agarose. The sample was purified by further agarose gel electrophoresis, electroelution and concentrated by passage through a Nacs prepac column. The purified fragment was redissolved in 20μl of water(1μg/μl).

(b) Interferon α2 sequence specific probes:

(i) 853 base pair fragment:

An 853 base pair DNA fragment containing the α2 interferon coding sequence and all the 3' non-coding region was purified from EcoRI digested pIF211. (Figure 6(c).) pIF211 (220µg) and 5µl EcoRI (100 units/µl) were incubated at 37°C for 2.5 hours in EcoRI incubation buffer. The 3 DNA fragments (107, 853 and 3659 base pairs) generated by the digestion were separated on a 7% polyacrylamide TBE gel. The 853 base pair fragment was further purified by electrophoresis on TAE agarose gels (1-1.5%), electroeluted and concentrated by passage through a Nacs prepac column. The sample was redissolved in 25µl water.

(ii)193, 260 and 400 base pair fragments:

BglII digestion of the 853 base pair fragment produced 3 smaller species 193, 200 and 400 base pairs long. (Figure 6(c)). These species spanned the entire coding and 3' non-coding regions of the α2 interferon sequence.

Initially pIF211 (100µg), 6µl 2-mercaptoethanol, 25µl 5 x BglII salts (50mM Tris.HCl, 250mM NaCl, 50mM $MgCl_2$, pH7.5), 10µl BglII (2 units/µl) and 59µl water were incubated overnight at 37°C. The pH of the sample was lowered by the addition of 20µl 5 x EcoRI incubation buffer (500mM Tris.HCl, 250mM NaCl, 50mM $MgCl_2$, pH7.5), then 4µl EcoRI (80 units/µl) was added and the sample incubated at 37°C for 3 hours. The DNA fragments were purified by electrophoresis on TAE agarose (1-1.0%) gels, electroeluted and concentrated by passage through a Nacs prepac column. Samples were redissolved in 25µl water.

(c) DNA fragments purified from pBR322/pAT153:

(i)346 base pair fragment:

A BamHI/HindIII fragment, 346 base pairs long was purified from pBR322 and pAT153. (Figure 6(d).) pBR322 or pAT153 (100µg), 5µl BSA (2mg/ml), 20µl 5 x BamHI incubation buffer (50mM Tris.HCl, 500mM NaCl,

25mM $MgCl_2$, pH8.0), 5µl BamHI (10 units/µl), 5ul HindIII (11 units/µl) and 35µl water were incubated at 37°C overnight. The resulting two fragments were separated on a TAE 1% agarose gel. The 346 base pair fragment was further purified by agarose gel electrophoresis, electroelution and concentrated by passage through a Nacs prepac column. Samples were redissolved in 25µl of water.

(ii) 692 base pair fragment:

An AhaIII 692 base pair fragment was purified from pBR322 and pAT153. (Figure 6(d).) pBR322 or pAT153 (50µg), 12.5µl 100mM 2-mercaptoethanol, 25µl 5 x AhaIII incubation buffer (50mM Tris.HCl, 250mM NaCl, 50mM $MgCl_2$, pH7.5), 10µl AhaIII (2 units/µl) and 52.5µl water were incubated at 37°C for 3 hours. Digestion with AhaIII generated 3 fragments 19,692 and 3652 (pBR322), 2947 (pAT153) base pairs long. They were separated on a TAE 1% agarose gel, the 692 base pair fragment purified by further agarose gel electrophoresis, electroelution and concentrated by passage through a Nacs prepac column. Samples were redissolved in 25µl of water.

4. Growth rate determinations in M9CA,MOPs glucose and polypeptone glucose media:

Starter cultures were grown overnight in liquid L-broth medium as described in General Methods 1. Overnight cultures (100µl) were added to 10ml of M9CA, MOPS glucose (Appendix B) or polypeptone glucose (Appendix B) media all containing trp (40µg/ml) to repress interferon production. These cultures were grown overnight to stationary phase then diluted 1:10 into fresh medium containing no trp, and grown for 90 minutes at 37°C before the addition of IAA (10µg/ml). Samples (0.5ml) were withdrawn from the growing cultures at appropriate time intervals and the absorbance at 650nm measured. From these measurements growth curves were obtained and the gradients, which represent growth rate, calculated.

5. Manipulations of rho-, temperature sensitive, HD 152 and isogenic rho+ KH54 E.coli strains:

(a) Selection of trp+ revertants:

HD 152 and KH 54 (75µl glycerol/L-broth culture) were inoculated into L-broth (10ml) and grown overnight at 30°C. On to M9CA plates containing thymidine, threonine, histidine, tyrosine, methionine, isoleucine and valine (all 40µg/ml) HD 152 (100µl overnight culture) and KH 54 (100µl overnight culture) were spread. The plates were incubated at 30°C overnight and trp+ colonies selected.

(b) Characterisation of HD 152 trp+ strain:

When HD 152 (containing the temperature sensitive rho- mutation) was grown at 30°C in polypeptone-glucose medium the number of viable cells increased exponentially. However, when the cultures were shifted from 30°C to 42°C the number of viable cells decreased after 1.5-2 hours, whereas the optical density measured at 650nm increased for the first 2-5 hours then remained constant. Viable cell numbers were determined after cultures (100µl) and a dilution series, 10^{-1} -10^{-6}, (100µl) were plated onto polypeptone plates and incubated overnight at 30°C

KH 54 trp+ was able to grow successfully at 30°C and 42°C.

(c) Transformation of α1 and α2 interferon plasmids into HD 152 trp+ and KH 54 trp+:

Competent HD 152 trp+ and KH 54 trp+ cells were prepared as described in General Methods 11. Plasmid DNA (20ng) was added to competent cells (HD152 trp+ and KH54 trp+) which were transformed as described (General methods 11). The response of all HD 152 trp+ strains containing the plasmids to a shift in growth temperature from 30°C to 42°C was determined.

6. Oligonucleotide-directed site-specific mutagenesis:

(Morinaga et al. 1984.)

(a) Production of oligomers:

Two synthetic oligomers (30 mers) were synthesised in an attempt to mutate the region 91 to 151 (co-ordinates Goeddel et al. (1981) of the α2 interferon sequence. In this region 4 arginine residues are encoded by rare E.coli tRNAs (AGG-3,AGA-1). The two arginine residues that occur together in each of the oligomers were replaced by the most commonly used E.coli arginine codon, CGT. (Oligomer 1;
AGCCTGGGTAGCAGGAGGACCTTGATGCTC.Oligomer 2;
CTGGCACAGATGCGTCGTATCTCTCTTTTC.)

(b) Preparation of DNA fragments:

(i) Pst I digested pIF213:

Plasmid pIF213 was digested with PstI (pIF213 (16.6µg), 3.75µl BSA (2mg/ml), 15µl 5 x HindIII incubation buffer (50mM Tris.HCl, 250mM NaCl, 50mM $MgCl_2$, pH7.6), 2µl PstI (10 units/µl) and water to a final volume of 75µl were incubated at 37°C overnight). After PstI digestion, 4µl 140mM 2-mercaptoethanol, 5µl 4mM dGTP, dTTP, dATP, dCTP and 5µl Klenow fragment of DNA polymerase I (2units/µl) were added and the samples incubated at room temperature for 1 hour. Filling in the cohesive ends generated by PstI digestion produced, on religation, a construct that was ampicillin sensitive. Alternatively, plasmid pIF213 linearised at the PstI site was treated with 2µl calf intestinal alkaline phosphatase (22 units/µl) for 1 hour at 37°C. Fragments subjected to phosphatase treatment were unable to religate.

Plasmid pIF213 digested with PstI and further treated with either Klenow or phosphatase was purified by phenol extraction, chloroform extraction and ethanol precipitation (2 volumes of ethanol and 0.1 volumes of 3M sodium actetae (pH5.5)). The samples were redissolved in 100µl water to give a final concentration of 0.1µg/µl. (Fragment 1.)

(ii)HindIII/PvuII double digest of pIF213:.

Plasmid pIF213 was digested with HindIII and PvuII. pIF213 (24.9µg), 3.75µg BSA (2mg/ml), 3.75µg 140mM 2-mercaptoethanol, 15µl 5 x PvuII incubation buffer (30mM Tris.HCl, 300mM NaCl, 30mM $MgCl_2$, pH7.4), 3.5µl HindIII (11 units/µl), 8.5µl PvuII (10 units/µl) and water to a final volume of 75µl were incubated at 37°C overnight. The two DNA fragments generated in the digest were purified by agarose gel electrophoresis (1-1.5% agarose), electroelution and concentrated by passage through a Nacs prepac oolumn. The small fragment contained the promoter region of the tetracycline gene and 380 base pairs of the α2 interferon sequence. The large fragment, Fragment II, was redissolved in 100µl of water to give a final concentration of 0.1µg/µl and was used in the subsequent mutagenesis reaction. Self ligation of this fragment produced a construct that was ampicillin resistant but tetracycline sensitive.

(c) 5'-Phosphorylation of the oligomers:

(Maxam and Gilbert 1977.) Oligomer 1 or 2 (200ng), 3µl 10 x kinase buffer (500mM Tris.HCl, 100mM $MgCl_2$, pH7.5), 3µl 10mM spermidine. 3µl 200mM dithio threitol, 6µl BSA (1mg/ml), 4mM dATP, 3µl polynuclease kinase (5.5 units/µl) and 9µl water were incubated at 37°C for 1 hour.

The samples were then heated to 85°C for 15 minutes, 2µl of 10mM ammonia added and the samples precipitated overnight at -20°C in 2 volumes of ethanol and 0.1 volume of 3M sodium acetate (pH5.5). After centrifugation (JA20 rotor, 12,000 rpm, 10 minutes -10°C) and drying the oligomers were redissolved in 16µl water (12.8 pmoles/µl).

(d) Mutagenesis reaction:

Fragments I (Klenow or phosphatase treated) and II (0.1µg of each), oligomer 1 (12.5 pmoles) and/or oligomer 2 (12.5 pmoles), 5.6µl 0.1 x TE buffer (1mM Tris.HCl, 0.1mM EDTA, pH7.5) and 2µl 10 x polymerase-ligase buffer (1M NaCl, 65mM Tris.HCl, 80mM $MgCl_2$, 10mM 2-mercaptoethanol,

pH7.5) were incubated in a boiling water bath for 3 minutes to denature the DNA fragments. The mixture was cooled gradually, 30 minutes at 30°C, 30 minutes at 4°C and finally 10 minutes in an ice bath. After cooling, 4µl of dATP, dTTP, dGTP and dCTP (2.5mM each), 2µl 10mM ATP, 1µl Klenow fragment of DNA polymerase I (2 units/µl) and 1µl water were added and the samples incubated at 15°C for 2 hours. T4 DNA ligase (0.5µl,400 units/µl) was added and the samples incubated at 12.5°C overnight.

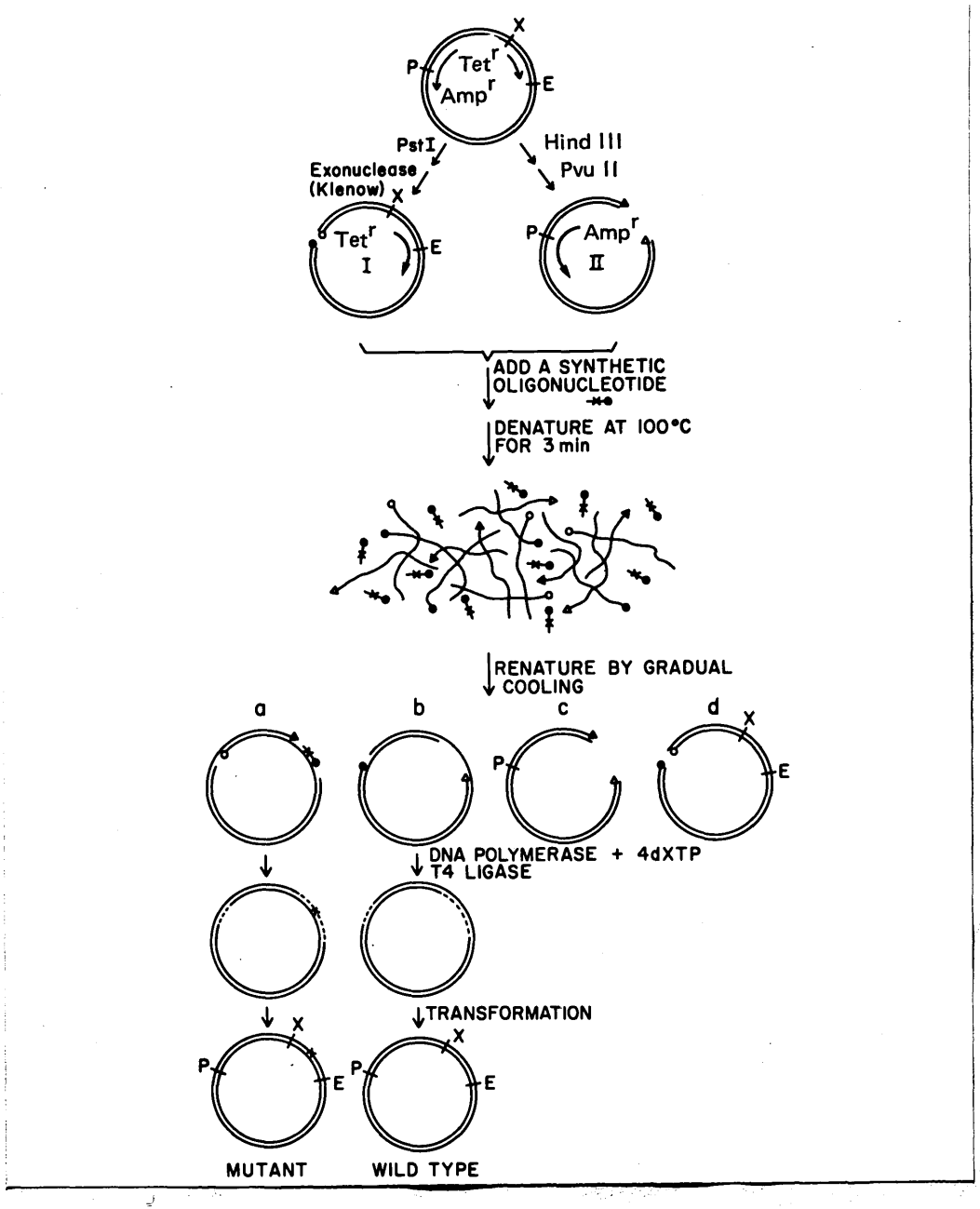
Transformation into E.coli DH1 yielded ampicillin and tetracycline resistant colonies only with oligomer 1. Transformants carrying the mutation were selected after RsaI digestion of small scale plasmid preparations since the incorporation of oligomer 1 into the interferon DNA sequence created an additional RsaI site. Plasmid pIF213 (10µl), incubated with RsaI (25 units) in RsaI incubation buffer (10mM Tris.HCl, 6mM $MgCl_2$, 14mM 2-mercaptoethanol, pH7.9) for 3 hours at 37°C produced three fragments, 245, 1094 and 2977 base pairs long. RsaI digestion of pIF213 containing oligomer 1 produced 4 fragments 76, 245, 1016 and 2977 base pairs in length. Visualisation of the digestion products on TBE 1% agarose gels under UV enabled the identification of the mutated plasmid.

The mutagenesis reaction is depicted diagramatioally in Figure 9.

7. Sanger dideoxy chain termination sequencing

Sanger et al. (1977) dideoxy chain termination sequencing was modified in an attempt to determine the precise site of termination of the rho-dependent terminator present in the α2 interferon coding sequence and the 5' region of the major pTH172 mRNA. Attempts were made to obtain both sequences using DNA fragments as template (KH211 mRNA) and primer (pTH172 mRNA). In addition conventional M13 templates were prepared and used in the analysis of the 3' end of the smallest prematurely terminating mRNA species produced by the α2 interferon constructions.

Figure 9: Diagrammatical representation of the site-directed mutagenesis of construction pIF213.



The schematic diagram depicts the steps involved in the site-specific oligodeoxyribonuoletide-directed mutagenesis of construct pIF213. (Adapted from Moringa et al. 1984.)

(a) Preparation of M13 templates:

The 193 base pair fragment obtained by EcoRI/BglII digestion of pIF211 was purified as described (Methods 3, (b), (ii)) and ligated into M13 mp19 digested with EcoRI and BamHI. (Digestion: M13 mp19 (1µg), 4µl 5 x BamHI incubation buffer (50mM Tris.HCl, 500mM NaCl, 25mM $MgCl_2$, pH8.0), 1µl BSA (2mg/ml), 0.5µl BamHI (12units/µl), 0.5µl EcoRI (12 units/µl) and 13µl water were incubated for 2 hours at 37°C. Samples were extracted with phenol:chloroform:isoamyl alcohol (25:24:1), then with chloroform and the aqueous phase precipitated in 2 volumes of ethanol and 0.1 volumes of 3M sodium acetate overnight at -20°C . After centrifugation (eppendorf bench centrifuge,5 minutes) the samples were dried in a rotary evaporator and resuspended in 50µl water.

Ligation: M13 mp19 BamHI/EcoRI (20ng), 193 base pair DNA fragment (3ng), 1µl 10 x ligase buffer (660mM Tris.HCl, 10mM EDTA, 100mM $MgCl_2$, pH7.2), 1µl 10mM ATP, 1µl 80mM dithiothreitol, 1µl T4 DNA ligase (400 units/µl) and water to 10µl were incubated at 4°C overnight.) The ligated samples were transformed into JM103. White plaques selected and templates prepared as described (Methods 3, (a), (i)).

(b) Sequencing using the Klenow fragment of DNA polymerase I:

Water (5µl), 1.5µl of RNA primer, 4µl of template and 1.5µl of H buffer (100mM Tris.HCl, 600mM NaCl, 66mM $MgCl_2$, pH8.0) were mixed at room temperature then incubated at 55°C for 3 minutes. It was left to cool to room temperature for 15 minutes and then placed on ice. $[\alpha^{35}S]$ dATP (600Ci/mmol) (2.5µl), 0.2M dithiothreitol (1µl) and Klenow fragment of DNA polymerase I (0.5 units) were added and the mixture split four ways, G (3µl), A (3µl), T (3µl) and C (3µl). To each of these was added 2µl of the appropriate deoxy/dideoxy mix (Table 1) and the samples were incubated at room temperature for 15 minutes. Chase reagent (2mM dGTP, dCTP, dTTP, dATP) (1µl) was then added to the samples which were left for a further 15

minutes at room temperature. Stop solution (100ml deionised formamide, 0.03g xylene cyanol FF, 0.03g bromophenol blue and 0.75g $Na_2EDTA.2H_2O$) (14µl) was added and the samples were incubated at 95°C for 3 minutes before loading (4µl) onto a 6% polyacrylamide, 7M urea gel. (For 100ml H_2O, 15ml 40% acrylamide (38g acrylamide, 2g N, N' methylenebisacrylamide in 100ml H_2O), 42g urea, 10ml 10 x TBE, 0.6ml 10g ammonium persulphate and 50µl TEMED.) The set gel was pre-electrophoresed at 40mA for 1 hour in TBE buffer to remove the ammonium persulphate and heat the gel. Samples were electrophoresed at 1200 volts for 2-3 hours.

Before autoradiography the DNA was "fixed" in 10% acetic acid for 10 minutes and then dried. Gels were autoradiographed for 3-7 days.

(c) Sequencing using Reverse Transcriptase:

The procedure used was similar to that using the Klenow fragment of DNA polymerase I except that RT buffer (50mM Tris.HCl, 30mM KCl, 10mM $MgCl_2$, pH8.3) was used in place of H buffer. Reverse transcriptase (24 units) was used to produce the complementary DNA strand and samples were incubated at 42°C to incorporate the label.

(d) Further purification of the RNA :

(i) Centrifugation through a caesium chloride cushion:

The RNA was resuspended in caesium chloride (0.4mg/ml) and placed carefully on top of 1.2ml of 5.7M caesium chloride in 0.1M EDTA, pH7.5. The sample was centrifuged, (ultracentrifuge, SW 50.1 rotor, 30,000 rpm, 19.2 hours at 20°C) the resultant RNA pellet resuspended in 350µl of water and then precipitated overnight at -20°C in 2 volumes of ethanol and 0.1 volume of 3M sodium acetate (pH5.5). The RNA was finally resuspended in 100µl of water after centrifugation (JA20 rotor, 12,000 rpm, 10 minutes, -10°C) and drying.

Table 1: Preparation of dNTP/ddNTP solutions for Sanger dideoxy sequencing.

Deoxy NPT mixes (A., C., T. and G.).

	A'	C'	T'	G'
0.5mM dCTP	20µl	1µl	20µl	20µl
0.5mM dTTP	20µl	20µl	1µl	20µl
0.5mM dGTP	20µl	20µl	20µl	1µl
1 x TE	20µl	20µl	20µl	20µl

Dideoxy working solutions.

0.1mM ddATP

0.1mM ddCTP

0.3MM ddGTP

0.5mM ddTTP

dNTP/ddNTP mixes.

A./ ddATP = 25µl A. + 25µl ddATP

C./ ddCTP = 25µl C. + 25µl ddCTP

T./ ddTTP = 25µl T. + 25µl ddTTP

G./ ddGTP = 25µl G. + 25µl ddGTP

(ii) Affinity Chromatography:

An attempt to separate the smallest prematurely terminating mRNA species, produced when the α2 interferon sequence is transcribed, was made using epoxy-activated cellulose. (Method of Moss et al. 1981). BRL epoxy-activated cellulose (50mg) was placed in a 1.5ml eppendorf tube and washed eight times with 1ml of 0.1M NaOH. Purified 260 base pair fragment obtained by EcoRI/BglII digestion of pIF211 (Methods 3, (b), (ii)) (20µg) and 50µl 0.1M NaOH was added to the cellulose. The tube was vortexed and then the contents placed on a sterile microscope slide in a high humidity chamber for 8 hours. The slide and contents were left for a further 2 hours on the bench top before the DNA-cellulose was washed with three 1ml washes of water. The epoxy groups remaining after the coupling reaction were inactivated in 1ml of 2M ethanolamine (pH9.0). The ethanolamine was removed by continued washing in water and the DNA-cellulose stored as an ethanol slurry at -20°C. Before use the resin was washed six times with 1ml of low salt buffer (10mM Tris.HCl, pH7.0, 90% formamide) and six times with 1ml of high salt buffer (0.6M NaCl, 10mM ribonucleoside-vanadyl complex, 10mM Tris.HCl, pH7.0). KH 211 total RNA (400µg) was redissolved in 100µl of high salt buffer and hybridised to the DNA-cellulose at 65°C for 24 hours. The unbound RNA was recovered by washing the cellulose in high salt buffer (0 x 0.5ml) and precipitating the species in 2 volumes of ethanol, overnight at -20°C. The bound α2 interferon mRNA were eluted from the column in 4.5ml of low salt buffer. Yeast transfer RNA (100µg) was added as carrier and the mRNA were precipitated in 2 volumes of ethanol and 0.1 volumes of 3M sodium acetate (pH5.5). Samples were centrifuged (JA20 rotor, 12,000 rpm, 10 minutes, -10°C), dried and resuspended in 100µl, ribosomal and small α2 interferon mRNA, 20µl other α2 interferon mRNA species.

The mRNA produced by pTH172 was also purified by epoxy-activated cellulose. The 367 base pair threonine attenuator sequence (20µg) was

coupled to the resin as described above. Total pTH172 RNA (500µg) was hybridised to the affinity column and interferon mRNA bound.The bound mRNA were eluted from the column in 4.5ml of low salt buffer, carrier, yeast transfer RNA (100µg) added and the samples precipitated overnight at -20°C in 2 .5 volumes of ethanol and 0.1 volumes of 0.3M sodium acetate (pH5.5). After centrifugation (JA20 rotor, 12,00rpm, 10 minutes at -10°C) and drying (rotary evaporator) samples were resuspended in 50µl of water.

III RESULTS AND DISCUSSION

A. The relationship of mRNA synthesis and stablity to expression of α1 interferon by different plasmid/E.coli constructs:

1. Copy number and expression levels:

Plasmid copy numbers were obtained for all four constructions to determine whether insertion of the foreign sequences had any affect on plasmid replication. Table 2 shows that inclusion of the α1 interferon sequence in both plasmids pBR322 and pAT153 resulted in increased copy number (two fold) over the parental plasmid when they were present in E.coli strain MM294. Twigg and Sherratt (1980) reported that the deletion that produces the pAT153 vector from pBR322 enables 1.5-3 times as many copies of pAT153 per cell when the plasmid copy numbers of these two vectors were compared. Introduction of the α1 interferon sequence did not alter this ratio.

The interferon sequence is promoted from the trp promoter and as such is inducible. Induction of interferon production results in a very slight increase in plasmid copy number per cell for all constructions (Table 2).

The four α1 interferon plasmids produce differing amounts of interferon as determined by bioassay of S100 lysates prepared from exponentially growing cultures (Table 3). Analysis of total cell protein on 12.5% polyacrylamide gels (Laemmli, 1970) confirmed these results (Figure 10). When interferon production is induced in these plasmid containing cultures the amount of interferon produced per plasmid copy is identical for pGW17 and pIF117 and similar for pTH171 and pTH172. The fact that pGW17 and pIF117 produce the same amount of interferon per plasmid copy is not suprising as these two plasmids differ only with respect to the deletion that produces pAT153 from pBR322.

Plasmids pTH171 and pTH172 both contain the threonine operon attenuator region inserted in opposite orientations immediately after the α1 interferon sequence. In plasmid pTH172 the attenuator is placed in the functional orientation while in pTH171 the sequence is inserted in the

Table 2: Determination of the plasmid copy number of each of the interferon α1 constructions under repressed and induced conditions.

Plasmid	Relative copy number	
	Repressed	Induced
pBR322	1	N/A
pGW17	1.6	2.0
pTH171	1.7	1.9
pTH172	1.9	2.5
pAT153	2.4	N/A
pIF117	2.9	4.2

Copy numbers were determined from an average of five separate determinations as described in Methods 1. and expressed relative to pBR322.

N/A indicates not applicable.

Bioassay:

Antiviral activity was determined by means of cytophathic stain-uptake inhibition in V3 cells infected with Semliki Forest Virus.

The specific activity of α1 interferon was 3.5×10^7 units/mg and α2 interferon 2×10^8 units/mg protein. (Rhodes et al 1986.)

Table 3: Quantitative analysis of α1 interferon production by different constructions under repressed and induced conditions.

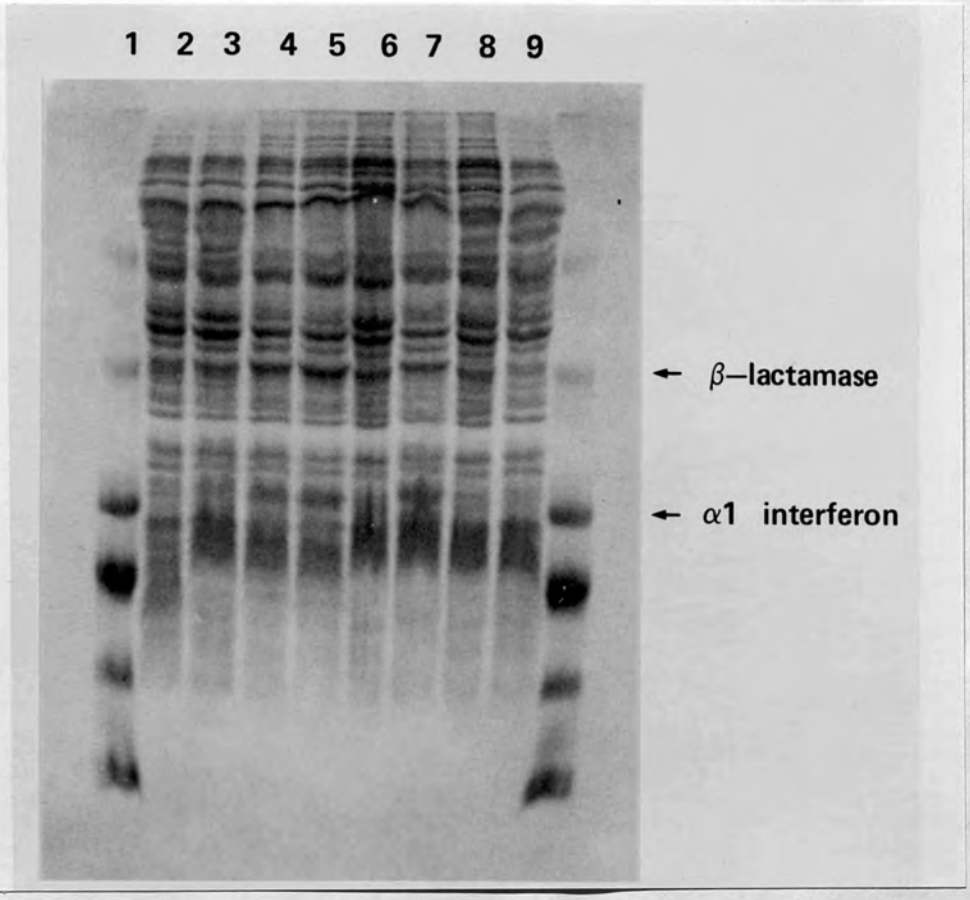
Source of interferon	Amount produced (mg/ml) culture			Amount/copy
	+trp	-trp	IAA	Induced
pGW17	1	2	4	2
pIF117	1.5	4	8	1.9
pTH171	n.t	3.5	8	4.2
pTH172	2.2	6	13	5.2

The protein production data represents the average of six separate determinations. The amount per copy was determined from copy number data expressed relative to pBR322.

n.t indicates not tested.

Induced indicates -trp +IAA.

Figure 10: Analysis of total cell protein produced by all the interferon α1 constructs.



Total cell protein (20μg) was loaded onto a 12.5% polyacrylamide gel and electrophoresised and stained as described in General Methods 9. Samples were isolated from cultures induced by trp starvation and IAA addition and additionally, in the case of pIF117, from repressed cultures (trp fed).

Lane 1, protein markers. Lane 2, pBR322. Lane 3, pAT153. Lane 4, pGW17. Lane 5, pIF117 (induced). Lane 6, pIF 117 (repressed). Lane 7, pTH172. Lane 8, pTH171. Lane 9, pWRL 50.

usually non-functional direction. Introduction of the attenuator in either orientation resulted in increased α1 interferon production. Cultures containing plasmid pTH171 were expected to produce similar quantities of α1 interferon as that obtained from pGW17 containing cultures because it was assumed that the attenuator sequence would have no affect when inserted in the non-functional direction.

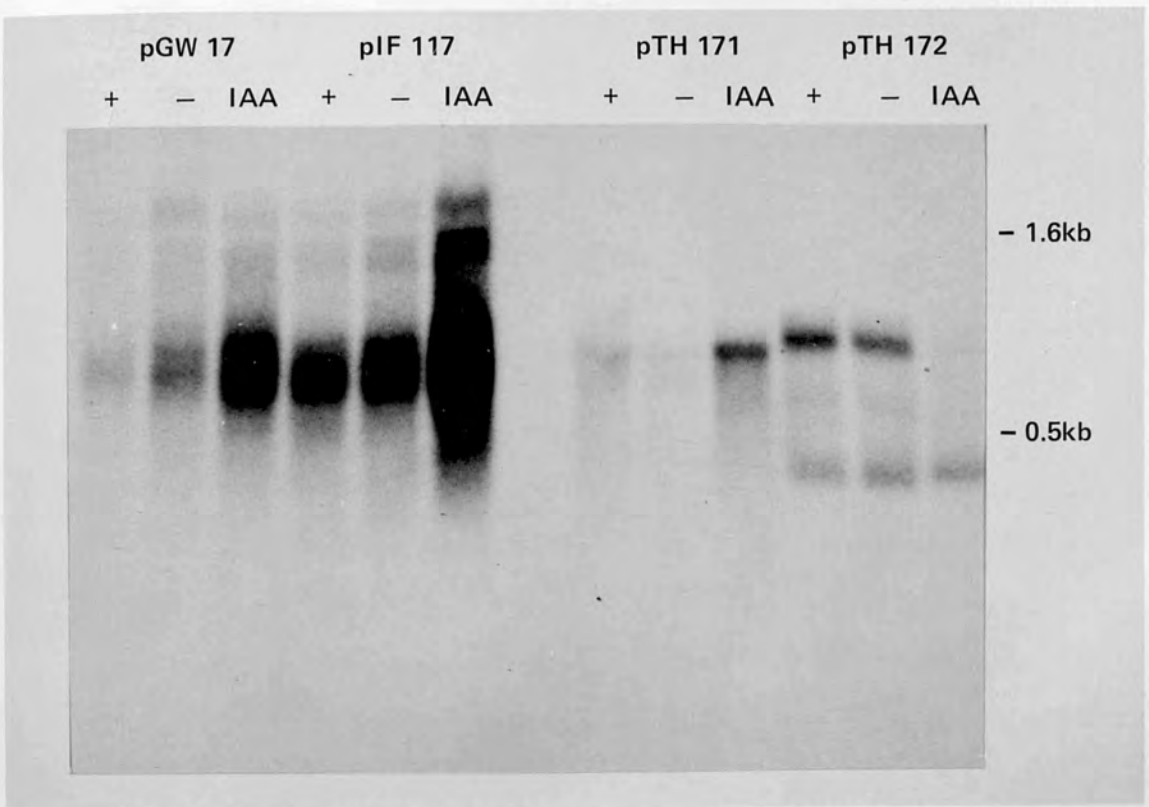
2. Analysis of the interferon mRNA species:

Analysis of the mRNA produced by all the constructions was undertaken in an attempt to determine whether interferon production was affected by message levels, message stability or translational efficiency.

Total RNA was isolated from cultures grown under three different conditions: (i) trp fed (ii) trp starved and (iii) trp starved and IAA induced, corresponding to repression, (i), and induction, (ii) and (iii) of the tryptophan promoter. The RNA extracted was blotted onto a Pall biodyne nylon membrane and then hybridised to a nick translated 676 base pair fragment (1.43×10^7 dpm/μg) corresponding to the entire α1 sequence. The autoradiograph obtained (Figure 11) revealed several interesting features. Maximal mRNA levels, corresponding to maximum induction of interferon production, (see Table 3) was obtained under conditions of trp starvation and IAA addition for three of the constructs but apparently not for pTH172. Under these conditions pTH172 produced only one major mRNA species while under conditions that gave rise to less interferon production more mRNA species were produced. Complete repression of the trp promoter was not obtained and induction by trp starvation alone resulted in only slightly increased mRNA levels. It is possible that repressor protein production for the chromosomal trp gene results in the formation of insufficient repressor-tryptophan complex to inactivate the trp promoter.

There were considerable differences in the mRNA species produced by the various constructs. pGW17 and pIF117 produced a multitude of mRNA

Figure 11: Analysis of interferon α1 mRNAs produced by different constructs under induced and repressed conditions.



Total cellular RNA (8μg) isolated from constructions pGW17, pIF117, pTH171 and pTH172 was probed with a nick translated interferon α1 DNA prepared as described in Methods 3, (a), (i),

The mRNA was isolated from cultures grown under three different conditions corresponding to; (+) repression of the trp promoter, (-) induction of the trp promoter by trp starvation and (IAA) induction of the trp promoter by trp starvation and IAA addition.

DNA size markers, 1.6 and 0.5 kilobases, are shown on the extreme right.

species. The species could be divided into two distinct groups; (i) those of approximately 600 base pairs in length and (ii) those of greater than 1.6 kilobase pairs in length. While the first group of mRNA were of the expected size the presence of the second group suggested that readthrough into the vector was occurring. Cultures containing pTH171 produced a single major mRNA species. This was unexpected since the only difference between pGW17 which produces multiple α1 interferon mRNA species and pTH171 which produces only one species is the introduction of the threonine operon attenuator immediately after the interferon sequence. The attenuator is inserted in reverse direction to that which gives rise to attenuation, thus mRNA species production is not affected by altering threonine concentrations in the growth media. In contrast, pTH172 which contains the threonine operon attenuator inserted in the correct orientation immediately after the interferon sequence produced two major mRNA species in the absence of IAA, the longer of which was most abundant. However, when the cultures were grown under conditions that produced most interferon ie trp starvation and IAA addition, pTH172 produced only one major mRNA species and that was the shorter less abundant one. When threonine was added to the medium containing trp and IAA to ensure that the attenuator was efficiently directing termination this single mRNA species was the major transcription product, the longer species was no longer evident. It appears that IAA is capable of mimicking the action of threonine as well as tryptophan. Although the mRNA species appears too small to encode for the complete protein nevertheless, translation of this species is most likely to give rise to the observed interferon protein.

There appears to be little correlation between the amounts of mRNA synthesised and the quantity of interferon produced. Construction pIF117. which produces the most mRNA produces only similar levels of interferon as those obtained from translation of the single pTH171 mRNA. Production of

most interferon is obtained from pTH172 which also produces one major mRNA species. This species may be more efficiently translated than its pTH171 counterpart. It is possible that mRNA stability may also be important in determining interferon production

3. Iniatiation of transcription:

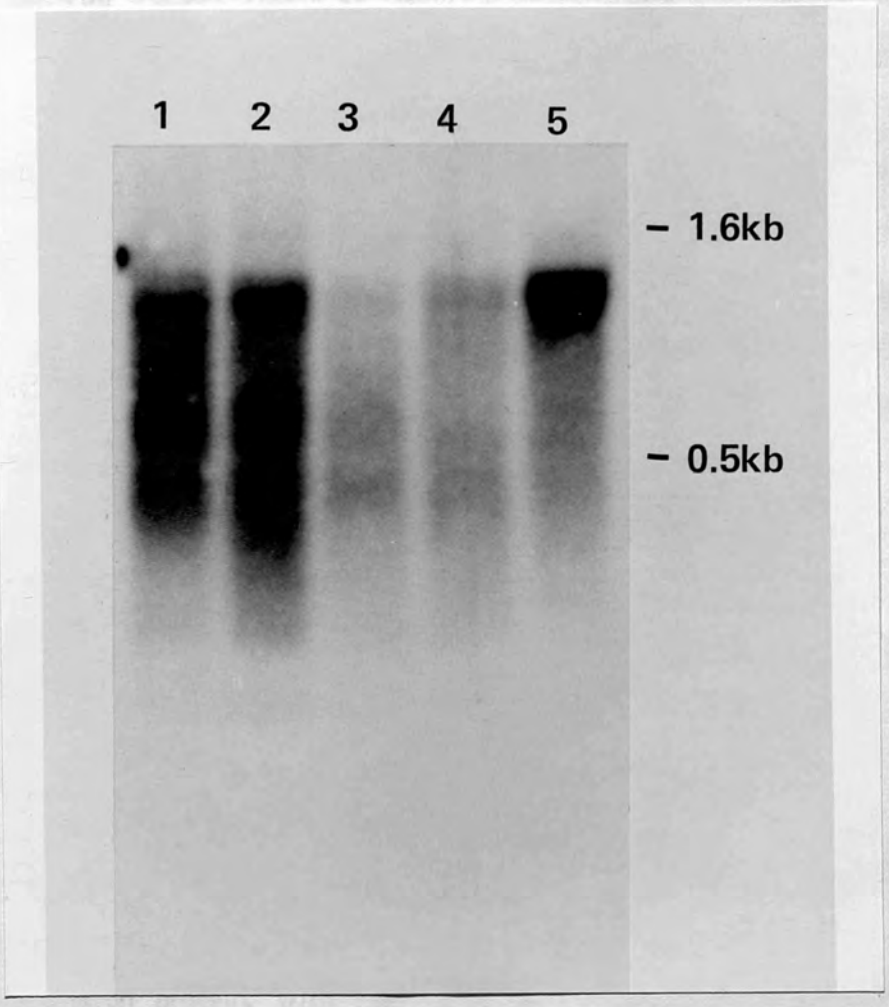
The multiple mRNAs produced by pGW17 and pIF117 could result from either initiation at other promotors or termination at many different sites or a combination of both. A known β-lactamase promoter, P3 (Brosius et al. 1982) initiates transcription in the same direction as the inserted trp promoter and is in close enough proximity to be considered as a likely alternative. To check that promotion was occurring at the inserted trp promoter and not from any intrinsic vector based sites a BamHI/HindIII fragment was isolated from pBR322. This BamHI/HindIII fragment was labelled with $[\alpha^{32}P]$ dATP (1.75×10^7 dpm/μg) and used to probe the RNA from all four constructs and from pBR322. Figure 12 shows that the mRNA profiles for all samples were similar to pBR322 and therefore contained no interferon species as the interferon probe did not hybridise to any pBR322 mRNA. The BamHI/HindIII fragment also contains the P2 promoter (Brosius et al. 1982) which initiates transcription of the tet gene and therefore the mRNA profiles shown in Figure 12 are likely to contain mRNAs for the tet protein(s) as well as β-lactamase.

The trp promoter appears to be the only promoter used to initiate transcription of the interferon gene. (The response of interferon production to trp induction (Table 3) provides further evidence that transcription is under trp promotor control.) Therefore, the mRNA species produced by pGW17 and pIF117 must result from utilisation of different termination sites either in the 3' non-translated or vector sequences.

4. Transcription termination of the interferon mRNA species:

Total RNA from the IAA-induced plasmid containing strains and

Figure 12: Detection of upstream promotion of interferon mRNA from all the constructs by α1 interferon.



Total cellular RNA (8μg) isolated from each of the interferon α1 constructions and pBR322 was probed with a nick translated HindIII/BamHI fragment prepared from pBR322 as described in Methods 3, (c), (i).

Lane 1, pBR322. Lane 2, pGW17. Lane 3, pIF117. Lane 4, pTH171. Lane 5, pTH172.

DNA size markers, 1.6 and 0.5 kilobases, are shown on the extreme right.

pBR322 containing strain was probed with radiolabelled fragments (302-1.03 x 10^7 dpm/µg, 88-2.5 x 10^7 dpm/µg, 172-7.3 x 10^6 dpm/µg, 114-1.0 x 10^7 d.pm/µg and 692-1.75 x 10^7 dpm/µg) in order to identify possible different termination sites. The small fragments of DNA were obtained by digestion of the interferon (302, 88, 172, 114 base pairs) and vector DNA (692 base pairs) sequences according to Figure 13(f). Four of the fragments (302, 88, 176 and 114 base pairs) contained only interferon sequence while the fifth (692 base pairs) contained part of the β-lactamase gene product. This fragment was selected because any long messages obtained from pGW17 and pIF117 were thought to result from inefficient termination at the end of the interferon gene. The RNA polymerase was thought to continue transcription into the next gene, β-lactamase, and to terminate at one or more of the β-lactamase termination sites (von Gabain et al. 1983).

The results are shown in Figure 13. The specificity of the interferon DNA probes is demonstrated by their failure to hybridise with mRNA from the pBR322 containing strain. (Figure 13 b, c, d and e.)

All four internal interferon probes recognised all the mRNA species produced by pGW17 and pIF117. (Figure 13 b, c, d and e.) Therefore all the mRNA produced by these two strains are capable of translation into mature interferon protein. With respect to termination, however, two distinct regions have been identified. As seen in Figure 11 both small and long mRNAs are present. The AhaIII probe hybridises with the longer mRNA but not with the smaller ones (Figure 13a). Therefore, termination of the smaller mRNAs must occur in the 3' non-coding interferon sequence or within the first few bases of the vector whilst the longer mRNAs readthrough into the β-lactamase gene. The likelyhood that these read through mRNAs also code for the β-lactamase protein is supported by polyacrylamide gel analysis of total protein showing increased levels of β-lactamase in pGW17 and pIF117 over pBR322 controls (Figure 10).

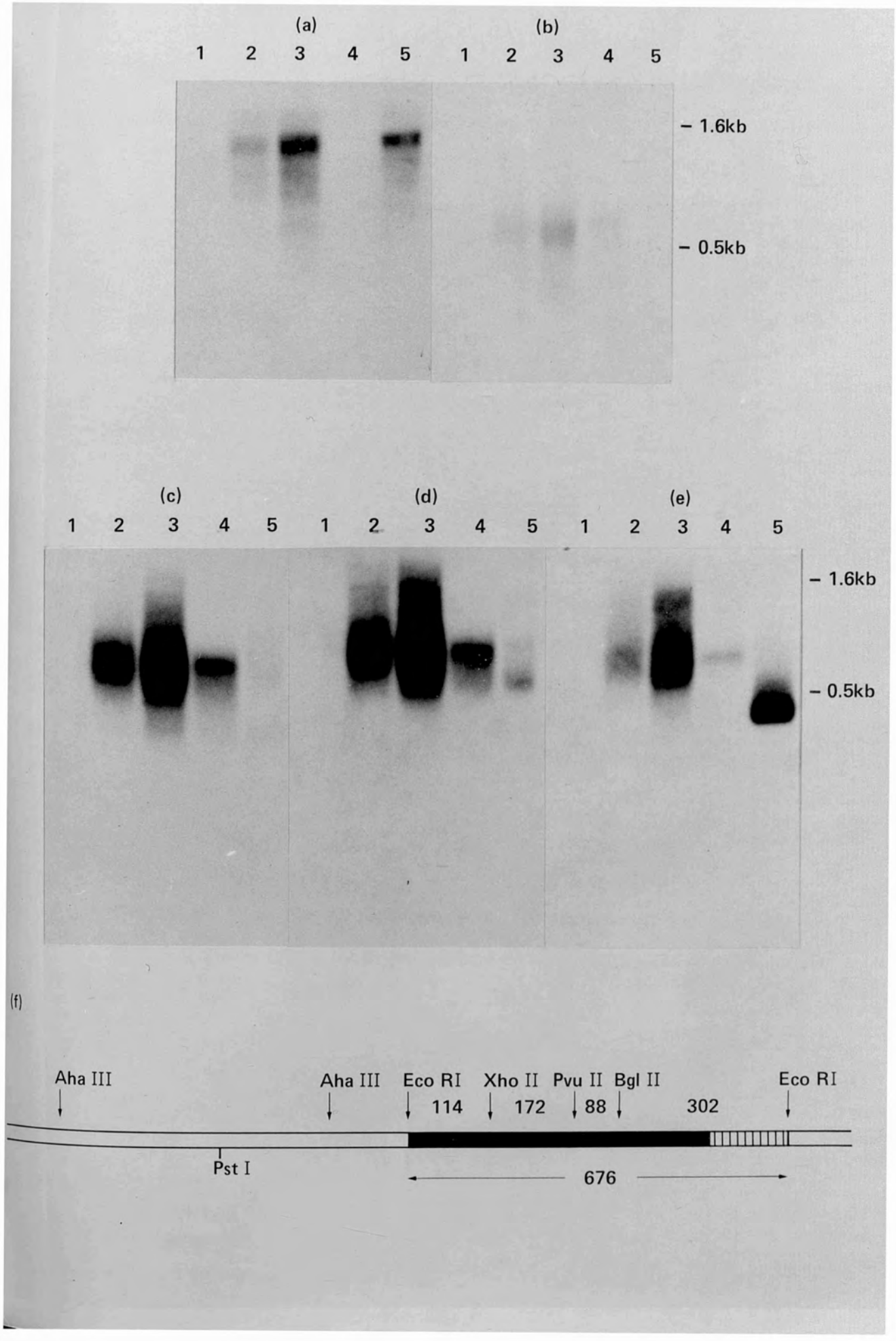
Figure 13: Comparison of termination sites used by the interferon α1 mRNA species.

Total cellular RNA (8μg) isolated from each of the interferon α1 constructions and pBR322 was probed with a series of fragments prepared as described in Methods 3, (a), (iii) and 3, (c), (ii).

Lane 1, pBR322. Lane 2, pGW17. Lane 3, pIF117. Lane 4, pTH171. Lane 5, pTH172.

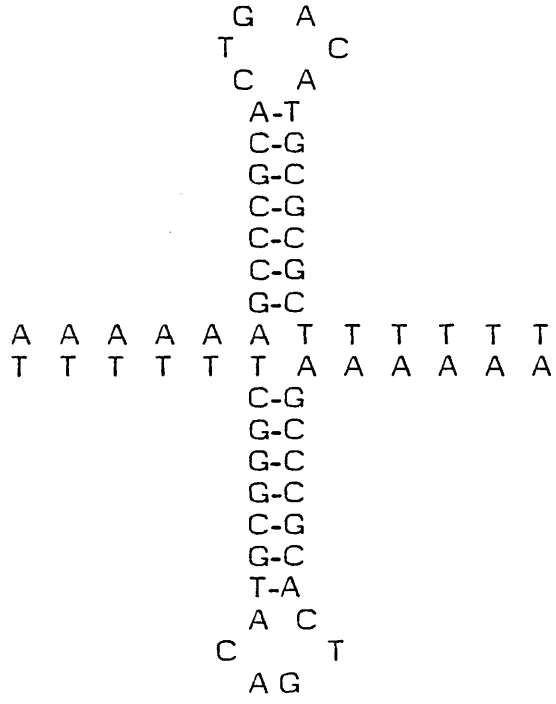
Panel (a) shows the constructs probed with a 692 base pair fragment obtained from AhaIII digestion of pBR322. The fragment contains the DNA between positions 3943 and 3251 as shown in Panel (f). The other panels are probed with fragments derived from digestion of the total EcoRI bound α1 sequence. This was cut with BglII, PvuII and XhoII at positions 258, 346 and 518 (Goeddel et al. 1981) respectively, to generate the 302, 88, 172 and 114 base pair fragments shown in Panel (f). Panel (b) 302 base pair fragment. Panel (c) 88 base pair fragment. Panel (d) 172 base pair fragment. Panel (e) 114 base pair fragment.

DNA size markers, 1.6 and 0.5 kilobases, are shown on the right of panels (b) and (e).


(a)
(b)
1 2 3 4 5 1 2 3 4 5
– 1.6kb
– 0.5kb
(c)
(d)
(e)
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
– 1.6kb
– 0.5kb
(f)
Aha III
Aha III
Eco RI
Xho II
Pvu II
Bgl II
Eco RI
114
172
88
302
Pst I
676

32827-8

Figure 14: Bidirectionally active terminiator derived from the threonine operon attenuator.



The figure shows the DNA sequence of the terminiator region of the threonine operon attenuator in the proposed stem-loop structure.

The major mRNA species produced by pTH171 was detected by all four interferon probes but does not appear to be a readthrough product since it failed to hybridise to the Aha III fragment. Termination of the pTH171 message appears to occur within the inserted threonine operon attenuator region at a discrete, very efficient site. This suggests that the threonine attenuator is an efficient terminator when inserted in either orientation ie. its function is bi-directional (Figure 14).

The mRNA produced by strains containing plasmid pTH172 yielded a most complicated series of results when probed with the interferon fragments. The major mRNA (Figure 11) was undetected by the 302 and 172 base pair interferon probes. Some minor species were detected by the 172 base pair probe and although faint recognition of the 88 base pair fragment was also observed the only fragment that detected the message unambiguously was the 114 base pair probe. While it is possible to explain the failure of the 302 base pair fragment to hybridise by suggesting that transcription of this message is promoted at a site further downstream - the message appears to be too small to code for the interferon protein- it is difficult to explain why this message does not hybridise to the intermediate 172 base pair fragment. One possible explanation is that this mRNA is much larger but forms an extremely stable secondary structure which is resistant to glyoxal and formaldehyde denaturation and therefore migrates anomalously in gels and is refractile to hybridisation by the 302 and 172 base pair probes. If this mRNA does arise from promotion at an internal interferon site it is difficult to explain why a similar species is not observed in any of the mRNA profiles from the other constructs.

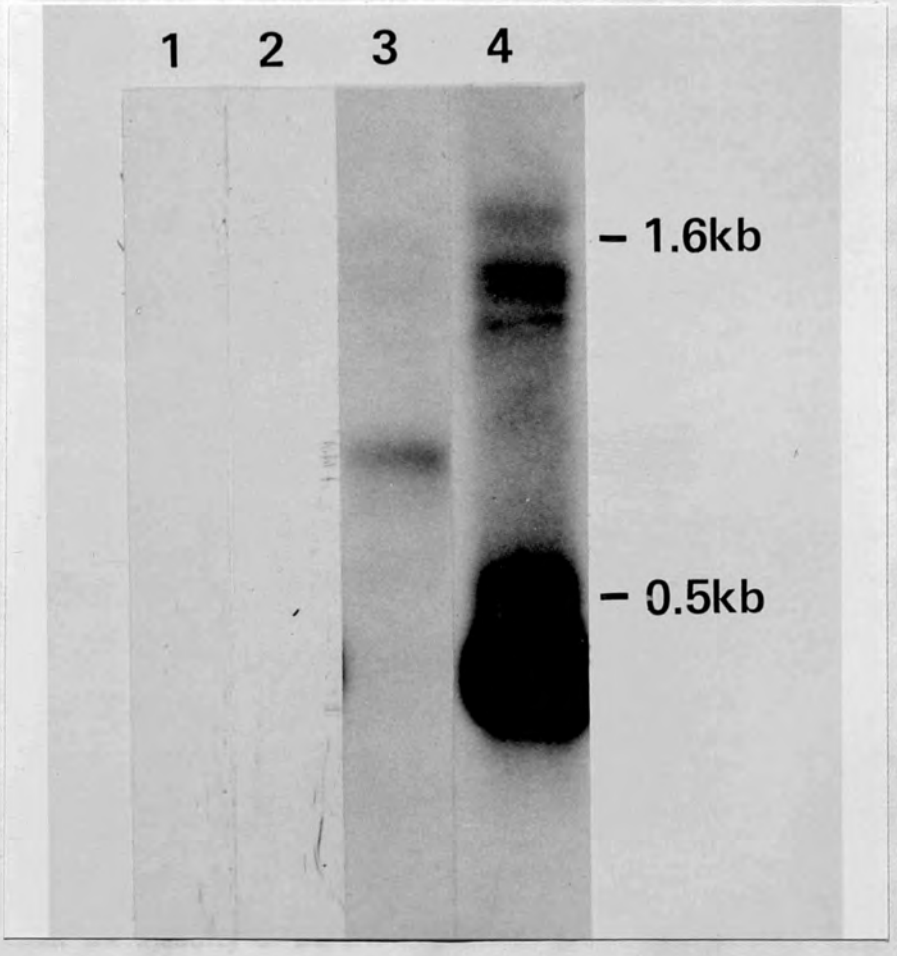
The 367 base pair fragment containing the threonine attenuator was purified, radiolabelled (2.1×10^7 dpm/µg) and used to probe mRNA from all four constructions. Figure 15 shows that only the strains containing pTH171 and pTH172 were detected by the probe. As expected mRNA produced by

pGW17 and pIF117 which do not contain this region were not recognised. Detection of the single pTH171 mRNA species by this probe confirmed that it terminated in the attenuator region. The mRNAs produced by pTH172 (Figure 11 track IAA) also appear to terminate in the threonine attenuator region since they hybridised with the threonine attenuator fragment but not the Aha III fragment. However, several longer mRNA species were detected with the AhaIII (Figure 13a) and attenuator probes (Figure 15) but not the interferon fragments (Figures 11 and 13b, c, d and e.) These may either represent minor interferon readthrough mRNAs not detected by the experimental conditions used or non-interferon mRNAs initiated from the threonine promoter. Nevertheless, in both pTH171 and pTH172, the threonine attenuator acts as the major site for termination of transcription and somehow prevents earlier termination in the 3' non-coding interferon sequence.

5. Half-lives of the interferon mRNA species:

Examination of mRNA intensities for the different constructs (Figure 11) would suggest, if there were a direct relationship between levels of transcription and protein production, that pIF117 and pGW17 produced more interferon than pTH171 and pTH172. However, the reverse is true suggesting that, at least in these cases, factors other than transcription are important in determining protein levels. The possibility that mRNA stability may be important in governing amounts of protein produced was investigated by examining the half-lives of the interferon mRNAs produced by the different constructs. Rifampicin (0.2mg/ml) was added to exponentially growing cultures induced by tryptophan starvation and IAA addition, blocking further initiation of transcription by RNA polymerase. At selected time intervals samples were removed and the RNA purified. The samples were probed with radiolabelled interferon α1 676 base pair fragment (7×10^6 dpm/μg). The results obtained (Figure 16) suggest that mRNA stability may play an important role in determining interferon levels. The major mRNA produced

Figure 15: Detection of interferon α1 mRNA containing the threonine operon attenuator by hybridisation to a fragment prepared from this region.



Total cellular RNA (8μg) isolated from each of the interferon α1 constructions was probed with a nick translated 367 base pair DNA fragment obtained by EcoR1 digestion of the threonine operon attenuator region of pTH172 as described in Methods 3, (a), (ii).

Lane 1, pGW17. Lane 2, pIF117. Lane 3, pTH171. Lane 4, pTH172. DNA size markers, 1.6 and 0.5 kilobases, are shown on the extreme right.

by construct pTH172 was stable for greater than 8 minutes while mRNA produced by pGW17 and pIF117 decayed uniformly with a half-life of approximately 1.5 minutes. pTH171 mRNA had an intermediate half-life of approximately 4 minutes. There appears to be a direct correlation between mRNA stability and levels of interferon production. Although pGW17 and pIF117 produce differing amounts of interferon but have similar mRNA half-lives, the higher interferon production by pIF117 may be explained by the increased levels of mRNA resulting from increased plasmid copy number.

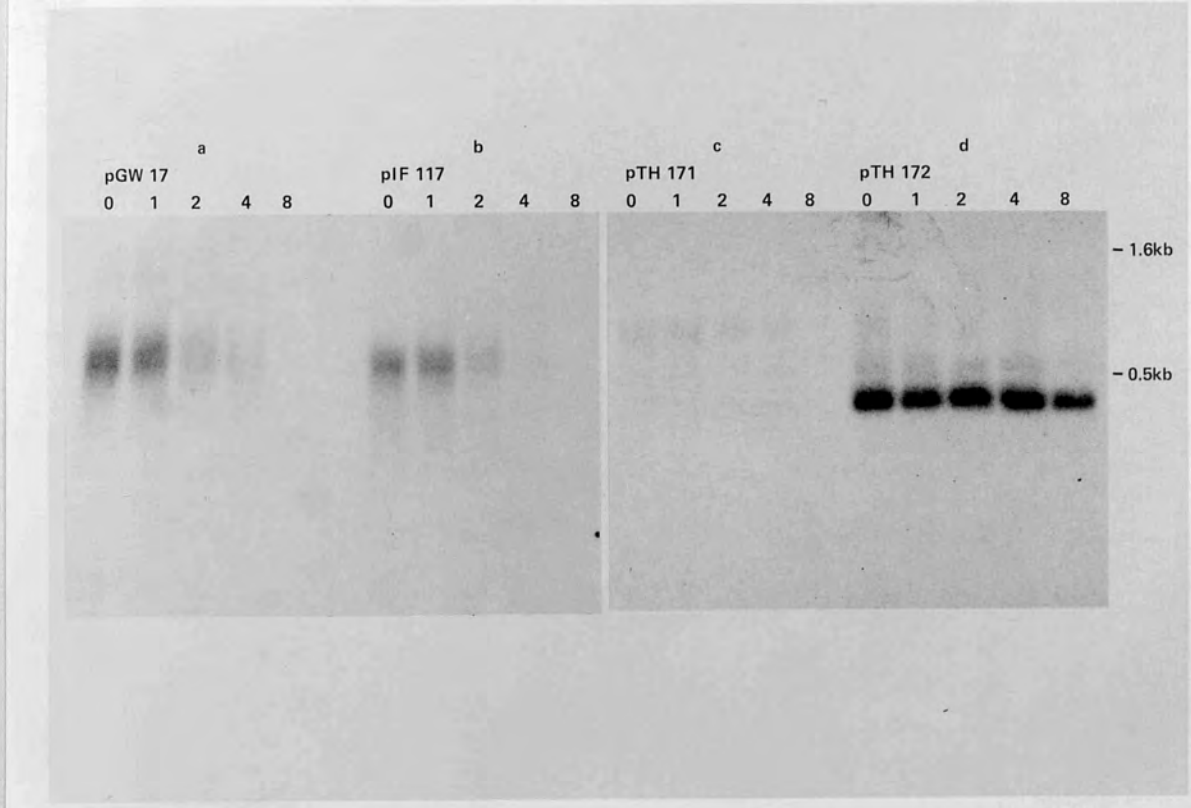
The multiple mRNA species produced by pIF117 appear individually more abundant than the species produced by pTH171 or pTH172, but they decay much more rapidly and their combined translation produced levels of interferon comparable to, and less than, those obtained for pTH171 and pTH172, respectively. Thus the addition and orientation of the threonine attenuator appeared to have a major affect on mRNA stability and interferon production.

6. Determination of the 5' end of the major pTH172 mRNA:

The major mRNA species produced by pTH172 was undetected by several of the interferon sequence specific probes particularly the 302 base pair fragment containing the 5' coding region (Figure 13). It was proposed that the inability of the pTH172 mRNA to be detected by various regions of the α1 sequence was a direct consequence of stable secondary structure formation by these regions. An attempt was made to determine the 5' end of the message to clearly establish whether or not the mRNA was initiated at the trp promoter and contained the trp initiation region. A modification to conventional Sanger dideoxy sequencing was used.

The RNA used in the sequencing reaction was prepared as described in General methods 5. (a) then further purified by affinity chromatography on epoxy-activated cellulose (Methods 7. (d), (ii)) containing the 367 base pair threonine operon attenuator bound to the resin. An 815 base pair TaqI

Figure 16: Comparison of interferon α1 mRNA half-live profiles of all the constructions.



Total cellular RNA (8µg) isolated at 0, 1, 2, 4 and 8 minutes after rifampicin treatment of constructions; (a) pGW17, (b) pIF117, (c) pTH171 and (d) pTH172 was probed with a nick translated 676 base pair DNA fragment corresponding to the entire interferon α1 sequence prepared as described in Methods 3. (a), (i).

DNA size markers, 1.6 and 0.5 kilobases, are shown on the extreme right.

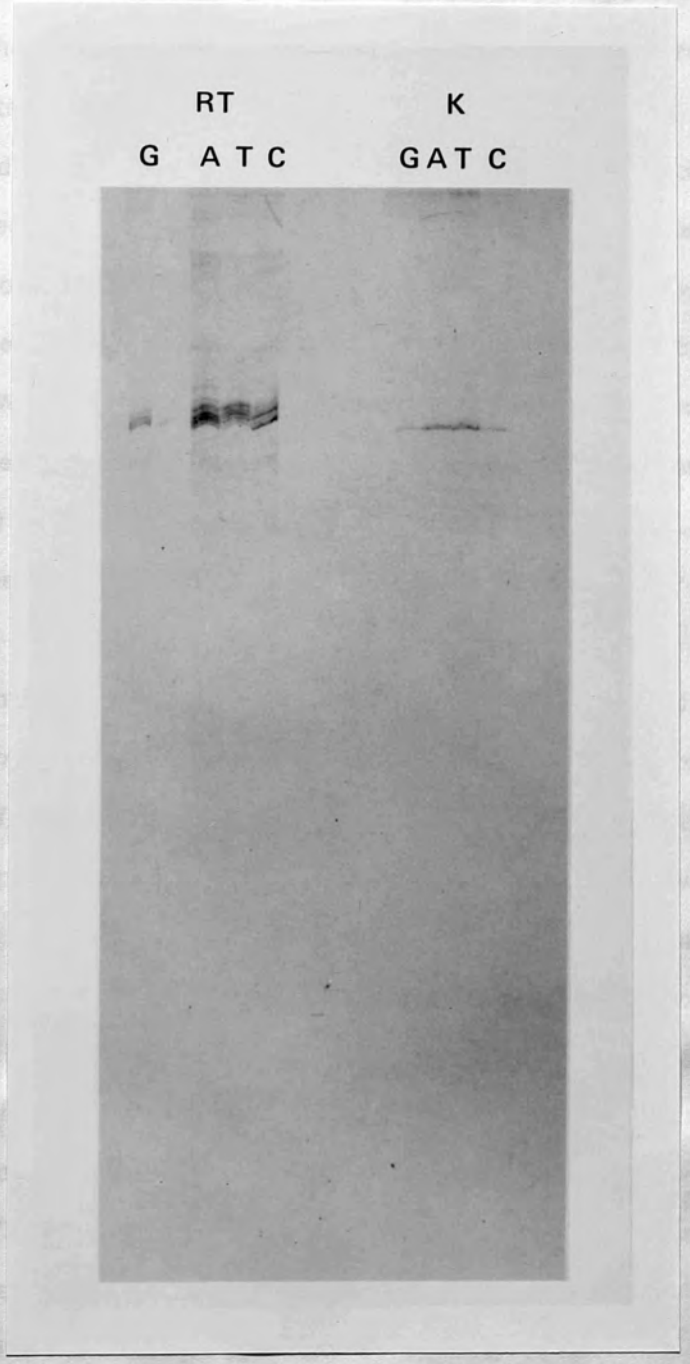
fragment (Methods 3, (a), (iv)) containing most of the α1 sequence and 246 base pairs of the threonine operon attenuator was purified and used as primer/template for sequencing the pTH172 mRNA. The TaqI fragment could be either primer or template for the sequencing reaction depending upon the site of promotion used by the pTH172 mRNA. If the mRNA was internally promoted the larger TaqI DNA fragment would act as template for elongation and the mRNA as the primer. If however, the mRNA was promoted from the trp promoter the TaqI DNA fragment would be shorter than the mRNA and act as a primer for the elongation reaction and the mRNA as template. While the Klenow fragment of DNA polymerase I is able to use only DNA as a template for elongation, reverse transcriptase can direct elongation on either an RNA or DNA template. Thus if the mRNA was internally promoted either enzyme could produce sequence data but if the mRNA was promoted from the trp promoter only reverse transcriptase would generate a sequence ladder.

Figure 17 shows the results obtained using (a) reverse Transcriptase and (b) the Klenow fragment of DNA polymerase I. Only a single species present in all four tracks was produced by the Klenow fragment. This is consistent with other artefactual observations (Bethesda Research Laboratories communication, Focus 1983) and supports that Klenow was unable to be used in this system. However, reverse transcriptase did appear to produce a number of radiolabelled DNA bands. Therefore, it would appear that the mRNA was extended at the 5' end beyond the TaqI fragment although the running conditions were inadequate to provide a precise sequence for the 20 nucleotides that are missing. The most likely conclusion is that the predominant pTH172 mRNA was initiated at the trp promoter and, indeed, the inability to hybridise with the α1 probes was due to the extremely stable secondary structure formed by this species.

Figure 17: Sequence analysis of the 5' end of the major pTH172 mRNA species.

pTH172 mRNA was annealed to the 815 base pair TaqI DNA fragment of interferon $\alpha 1$ and the sequence was extended by the method of Sanger et al. (1977) using (a) Reverse transcriptase and (b) the Klenow fragment of DNA polymease I.

The order of the tracks is the same for both (a) and (b).From left to right: G, A, T and C.


RT
K
G A T C
G A T C

7. Summary of results:

Production of α1 interferon varied appreciably in the four constructions examined. While pGW17 and pIF117 were expected to produce differing amounts of protein the increases produced by introducing the threonine attenuator were not expected.

Plasmid copy number studies showed that the introduction of the α1 interferon sequence into plasmids pBR322 and pAT153 increased the copy number. Constructions containing the α1 sequence had twice as many plasmid copies per cell as those without. The reason for this increase is not understood. When interferon production is expressed as mg produced per litre of culture per plasmid copy pGW17 and pIF117 produce similar quantities (2mg/l/copy) as do pTH171 (4.2mg/l) and pTH172 (5.2mg/l/copy). Addition of the threonine attenuator, regardless of orientation, increased interferon production.

The synthesis of multiple interferon mRNAs by pGW17 and pIF117 was unexpected but not unprecedented. von Gabain et al (1983) reported the production of six pBR322 encoded β-lactamase messages, resulting from the use of two promotors and three terminators. It has been shown that the multiple species produced by the constructions resulted from utilisation of many termination sites. The inserted interferon sequence while containing all the information required to code for the mature protein contained little noncoding sequence and no obvious termination sequences. Thus, although termination occurred at several places within the non-coding region it was not 100% efficient. This enabled the production of several long readthrough mRNA. These species terminated in the vector sequence at the β-lactamase terminators and the messages produced were polycistronic. The half-lives of both groups of messages were the same,1.5 minutes. None of the interferon producing constructs produced any detectable monocistronic β-lactamase message. Promotion of such a species might be expected from the intrinsic

P3 site (Brosius et al. 1982). It has been suggested that transcription through a promoter may inhibit its activity. Hausler and Somerville (1979), Adhya and Gottesman (1982) and Malan and McClure (1984) showed that promotion of a gene from several promoter sites in the same mutant yielded less protein than that obtained by summation of the individual contributions of each promoter. The interferon gene is promot ed from the strong trp promoter in the α1 interferon constructions and it is possible that transcription initiated from this site prevented or limited severely the RNA polymerase binding at the P3 β-lactamase promoter.

The threonine attenuator region was added after the interferon sequence to provide a known, efficient termination signal. Introduction of this region into the plasmid pGW17 was possible in two opposing orientations. The resulting plasmids were pTH171 and pTH172 with pTH172 containing the attenuator in the "correct" orientation. Transcription products obtained from these two plasmids showed that introduction of an efficient rho-independent termination site resulted in the production of one major mRNA species providing, in the case of pTH172, that threonine was included in the growth medium. The absence of threonine from the growth medium enabled the formation of the antiterminator structure and subsequent readthrough at the attenuator. Termination of mRNA produced by pTH171 was, as expected, not affected by cellular threonine levels. It appeared that the threonine attenuator was efficient at directing termination regardless of its orientation in the plasmid. Termination at the attenuator was more efficient, as judged by mRNA species production, if the attenuator was inserted in the "non-functional" orientation. Schollmeier et al. (1985) and Hudson and Davidson (1984) have reported on the bidirectional action of other transcription terminators. Examination of the sequence of the inserted threonine attenuator revealed that stable hairpin loop formation can occur in

either direction and that the loop is followed by a run of at least six Ts in both directions. (Figure 14.)

The anomolous behaviour of the major pTH172 mRNA on denaturing gels led to the investigation of its composition by differential probing. Probes were obtained corresponding to the complete length of the predicted pTH172 mRNA product. These were used to screen the pTH172 mRNA species and produced a complex set of results. Several of the fragments failed to hybridise to the major pTH172 mRNA species. One possible explanation for these results is that the mRNA was promoted from some internal α1 interferon coding sequence region. An alternative explanation is that the mRNA produced by pTH172 formed a secondary structure that was particularly resistant to denaturation. The mRNA half-life studies revealed that the pTH172 mRNA was indeed exceptionally stable with a half-life in excess of 8 minutes. Regions of mRNA involved in secondary structure base pairing would be unavaliable for interaction with the single stranded DNA probes and would be undetected. Attempts to determine the 5' end of the major pTH172 failed to produce any readable sequence but the size of the species into which counts were incorporated and the enzyme that catalysed that incorporation supported the assumption that transcription was initiated at the trp site.

Expression of interferon α1 in E.coli seem to be dependent on pre-translational influences.

B. The relationship of mRNA synthesis and stability to expression of α2 interferon in plasmid/E.coli constructs:

1. Copy number, expression levels and plasmid stability:

A series of plasmids containing the α2 interferon sequence was created in an attempt to maximise protein production. The plasmids were derived from a parental plasmid, pIF211, by a series of alterations to either the 5' or 3' non-coding regions. Plasmid copy number and interferon levels were

determined for each of the constructions. Table 4 shows that the α2 interferon sequence unlike the α1 sequence did not affect plasmid copy number. The four pAT153 based plasmids had copy numbers similar to pAT153 while pIF217 which is pBR322-based had a copy number identical to pBR322. Most interferon was produced by pIF217 which differed from all the other constructions in that it contained an altered ribosome binding site region and was on the lower copy number vector pBR322. Small differences in interferon production were observed between the other constructions, these correlated with the presence or absence of the region 5' to the -35 region of the trp promoter. The removal of this region resulted in decreased interferon levels. As with α1 interferon, maximum protein production was obtained from cultures that were induced by tryptophan starvation and IAA addition (Table 5).

A pAT153 based plasmid containing the altered ribosome binding site was created (pIF215) but it failed to produce detectable levels of α2 interferon. Plasmid stability studies (Table 6) showed that pIF215 was very unstable and that all pAT153 based α2 producing constructs were unstable to a greater or lesser degree. The cause of the instability is not clear as a 5 fold increase in interferon production (0.1mg/l, pIF212 to 0.5mg/l, pIF211) results in much greater plasmid instability (10%, pIF212 to 99%, pIF211). However, pBR322 based pIF217 and all the α1 interferon plasmids were 100% stable under the conditions tested. The unstable plasmids could be maintained by the inclusion of tetracycline in the growth medium.

2. Analysis of interferon mRNA species:

Analysis of the interferon transcription products was undertaken in an attempt to determine whether variations in protein production were attributable to transcription, translation or to some intermediate state similar to that obtained for the α1 interferon producing constructs.

Table 4: Determination of the plasmid copy number of each of the α2 interferon constructions under repressed and induced conditions.

Plasmid	Relative copy number	
	Repressed	Induced
pBR322	1	N/A
pIF217	1.0	1.1
pAT153	2.4	N/A
pIF210	1.9	1.6
pIF211	1.7	2.2
pIF212	2.0	2.1
pIF213	1.6	1.9

Copy numbers were determined from an average of five separate determinations as described in Methods 1. and expressed relative to pBR322.

N/A indicates not applicable.

Table 5: Quantitative analysis of α2 interferon production by the different constructions under repressed and induced conditions.

Source of interferon	Amount produced (mg/ml) culture		Amount/copy
	Repressed	Induced	Induced
pIF210	0.04	0.10	0.06
pIF211	0.12	0.50	0.23
pIF212	0.05	0.10	0.05
pIF213	0.023	0.50	0.26
pIF217	n.t	4.0	3.6

The protein production data presented represents the average of six separate determinations. The amount/copy was determined from copy number data expressed relative to pBR322.

n.t indicates not tested.

Table 6: Comparison of plasmid stabilities of all the different interferon constructs under repressed and induced conditions.

Plasmid	% Stable colonies	
	Repressed	Induced
pIF210	n.t	30%
pIF211	10%	1%
pIF212	100%	90%
pIF213	n.t	1%
pIF215	0.05%	0.001%
pIF217	100%	100%
pBR322	100%	100%
pAT153	100%	100%
all α1 plasmids	100%	100%

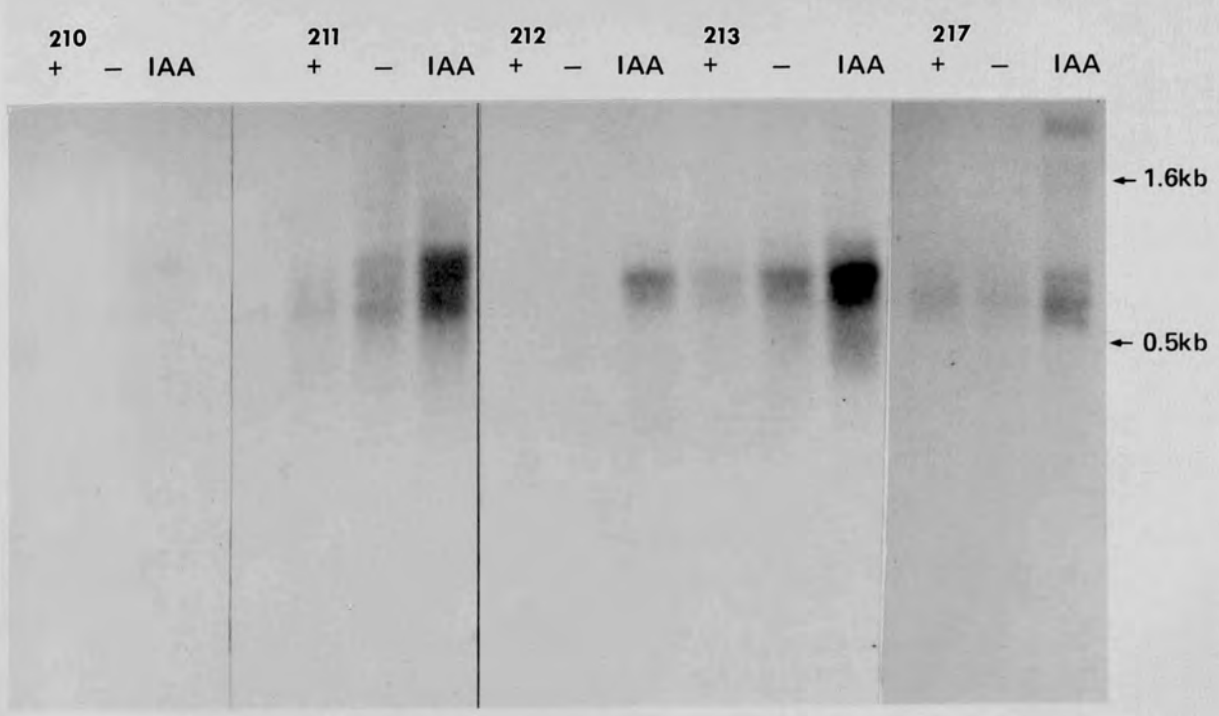
Plasmid stabilities were determined after growth in liquid media for 40 generations. The data presented represents the average of four separate determinations.

n.t indicates that these samples were not tested.

Figure 18 shows the interferon α2 mRNA produced by the constructions when (i) repressed, (ii) induced by trp starvation and (iii) induced by trp starvation and IAA addition. The mRNA was detected by hybridisation to an interferon α2 specific, 853 base pair fragment radiolabelled with $[\alpha^{32}P]$ dATP (7.7×10^7 dpm/μg). As seen, induction of interferon production by trp starvation and IAA addition produced most interferon mRNA. (No mRNA species were detected for constructs pIF210 and pIF212 when these constructs were repressed or induced, by trp starvation under the conditions used.) Induction by trp starvation and IAA addition gave rise to maximum protein production. As with the α1 interferon constructions there is very little difference in message production between the repressed condition, trp fed, and trp starvation in the absence of IAA and it appears that complete repression of the trp promoter is not possible when it is contained on such a high copy number vector.

Figure 19 shows the interferon mRNA obtained from maximally induced cultures of all the α2 interferon producers. Transcription of pIF210 and pIF212 is poor in comparison with the other constructs. They are also poor interferon producers. Constructs pIF210 and 212 lack the sequence 5' to the -35 region of the trp promoter. This region is obviously important for RNA polymerase binding and transcription initiation. As with the α1 constructions there is a multiplicity of mRNA species produced. The species produced by pIF212, pIF213 and pIF217 are identical differing only in quantity (Figure 19). Several of these species are also produced by pIF211 and pIF210. A longer exposure of pIF 210 produces a mRNA profile identical to that obtained for pIF211. All these constructions produce several minor mRNAs that appear too small to contain sufficient information for translation into mature α2 interferon. This is in contrast to the α1 case where although multiple mRNA species were observed for plasmids pGW17 and pIF117 all the species produced were long enough for translation to yield the mature α1 protein.

Figure 18: Analysis of interferon α2 mRNAs produced by all the different constructs under repressed and induced conditions.

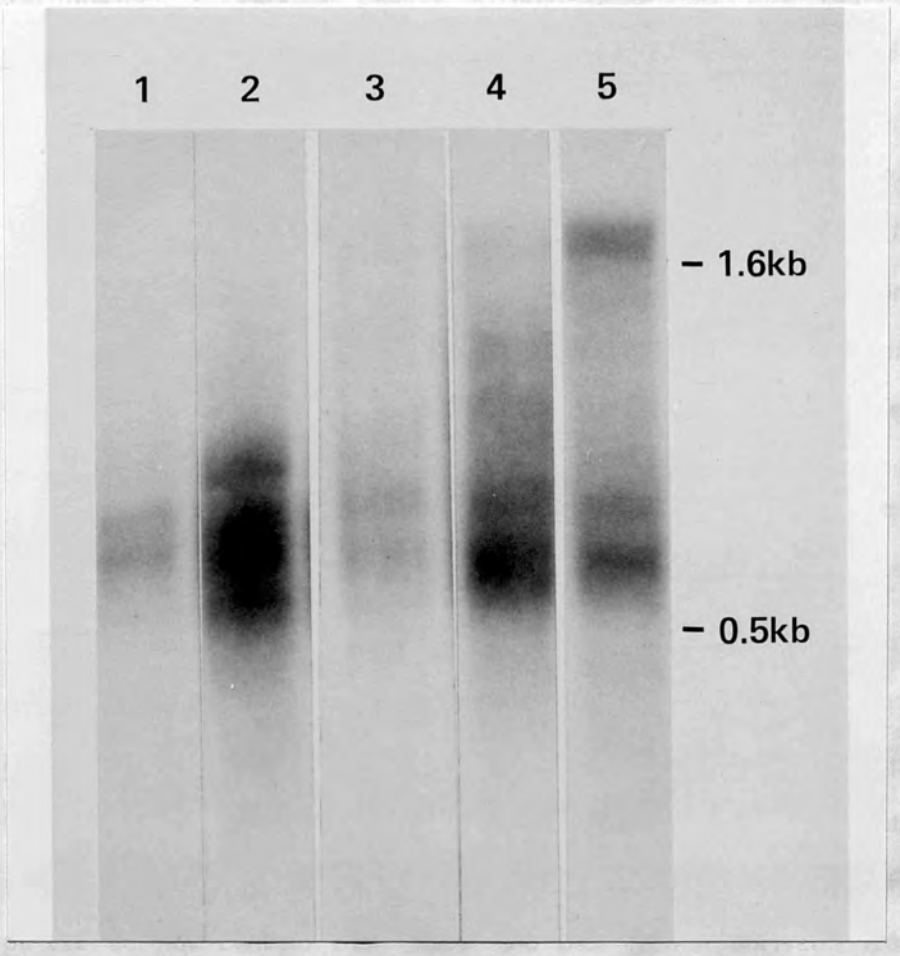


Total cellular RNA (8μg) isolated from constructions pIF210, pIF211, pIF212, pIF213 and pIF217 was probed with a nick translated 853 base pair interferon α2 DNA fragment prepared as described in Methods 3, (b), (i).

The mRNA was isolated from cultures grown under three different conditions corresponding to; (+) repression of the trp promoter, (-) induction of the trp promoter by trp starvation and (IAA) induction of the trp promoter by trp starvation and IAA addition.

DNA size markers, 1.6 and 0.5 kilobases, are shown on the extreme right

Figure 19: Comparison of the mRNA profiles of all the interferon α2 constructions.



Total cellular RNA (8μg) isolated from each of the interferon α2 constructions was probed with a nick translated 853 base pair interferon α2 DNA fragment prepared as described in Methods 3, (b), (i).

Lane 1, pIF210. Lane 2, pIF211. Lane 3, pIF212. Lane 4, pIF213. Lane 5, pIF217.

DNA size markers, 1.6 and 0.5 kilobases, are shown on the extreme right.

Plasmids pIF210 and pIF211 which contain the complete non-coding region of the interferon gene produce no very long mRNA species. Termination appears to occur efficiently within this region. Those constructions in which this long non-coding region has been deleted produce several very long mRNA species.

Although pIF217 has been shown to have a copy number half that of all the other α2 constructs (Table 4) it produced similar quantities of mRNA. The reason for this is not clear as the α1 interferon constructs contain the same ribosome binding site region but pGW17 and pIF117 which differ in copy number differ similarly in mRNA quantity. Perhaps the bases in the vicinity of the ribosome binding site contribute to promoter efficiency in some way.

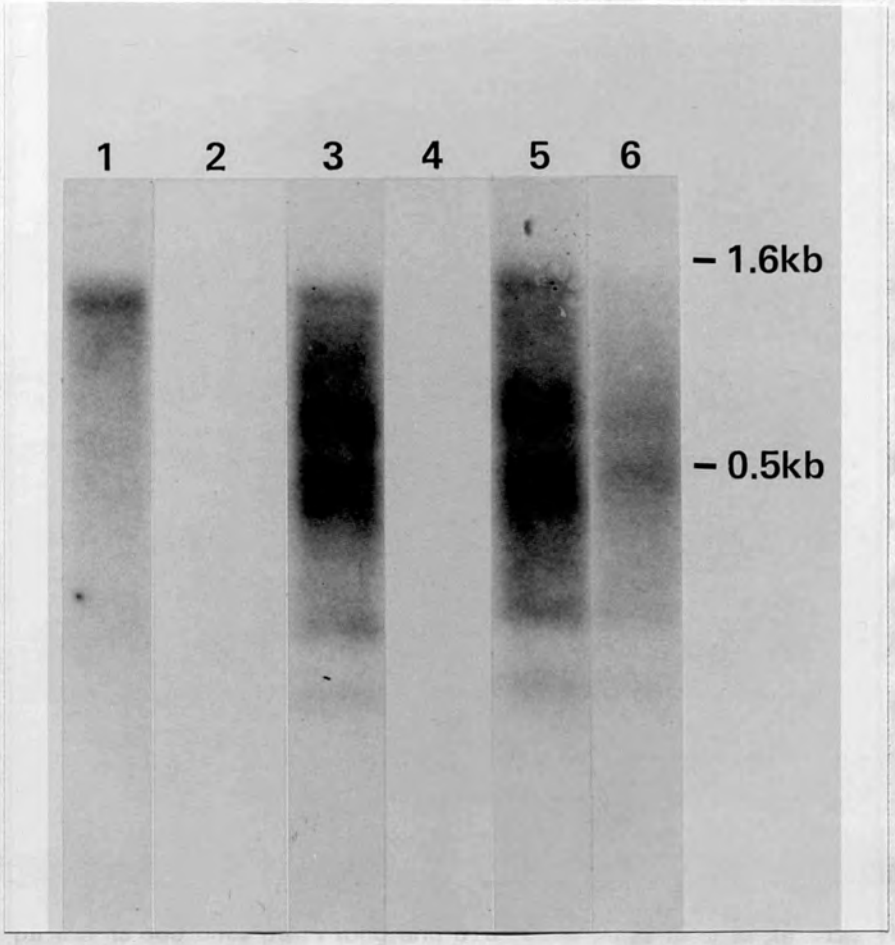
3. Initiation of transcription:

As with α1 interferon constructions pGW17 and pIF117 the multiple mRNA produced by the constructs may result from promotion at several sites, termination at several sites or a combination of both. The HindIII/BamHI fragment containing the intrinsic P3, β-lactamase promoter (Brosius et al. 1982) was used to probe the RNA from the constructs to ascertain whether or not the P3 promoter was used (Figure 20). pIF210 and pIF212 do not contain this region and thus mRNA derived from these constructs was not recognised by the probe (1.5×10^7 dpm/μg). The profiles obtained for pIF211, pIF213, pIF217 and pAT153 were similar. Thus as with the α1 interferon constructs it appears that transcription of interferon mRNA is initiated only at the trp promoter site. Induction of α2 interferon mRNA production by trp starvation and IAA addition is further evidence in support of this.

4. Transcription termination of interferon mRNAs:

The mRNAs that contain α2 interferon sequence may be divided into three distinct groups; (i) those that are very long, (ii) those that are produced by all the constructs and are of sufficient length to contain the entire α2 sequence, (iii) those that appear too small to code for the mature α2 protein.

Figure 20: Detection of upstream promoters of interferon mRNA from all the constructions by α2 interferon.



Total cellular RNA (8μg) isolated from each of the interferon α2 constructions and pAT153 was probed with a nick translated HindIII/BamHI fragment prepared from pAT153 as described in Methods 3, (c), (i).

Lane 1, pAT153. Lane 2, pIF210. Lane 3, pIF211. Lane 4, pIF212. Lane 5, pIF213. Lane 6, pIF217.

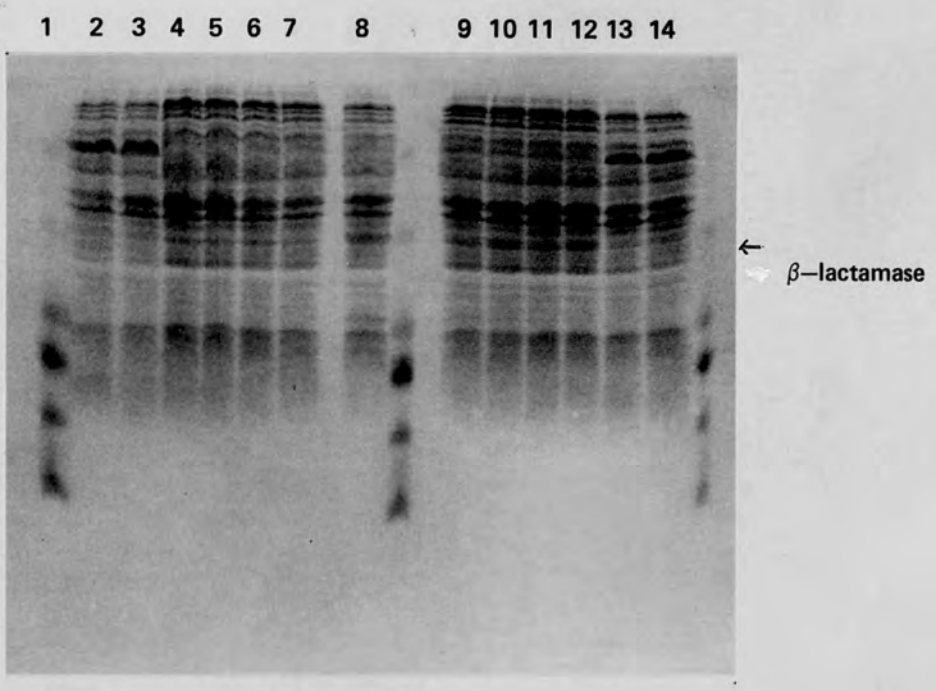
DNA size markers, 1.6 and 0.5 kilobases, are shown on the extreme right.

Polyacrylamide gel electrophoresis of total cell protein revealed an increase in β-lactamase expression for constructions pIF212, pIF213 and pIF217, which all produce the long mRNAs species (Figure 21). It is possible that these long mRNA are the result of inefficient termination at the end of the interferon sequence, enabling the polymerase to continue transcription into the vector. In order to establish whether these species were readthrough mRNAs and responsible for the observed increase in β-lactamase production a 692 base pair AhaIII fragment was purified from pAT153 and used to probe the mRNA from the α2 constructs and pAT153. As seen in Figure 22 no mRNA species produced by pIF210 and pIF211 are detected by this probe (1.5×10^7 dpm/μg). Apparently termination of the interferon messages occurs within the long non-coding region. The constructions that contain the shortened 3' non-coding region, pIF212, pIF213 and pIF217 all produce long mRNAs that are detected by the probe. Increased β-lactamase production was only observed in those constructions producing the long mRNAs suggesting that these species are polycistronic containing α2 interferon and β-lactamase messages.

The α2 interferon sequence contained in constructions pIF213 and pIF217 is 660 base pairs long and 616 base pairs long in pIF212. The size of these inserts compares very favourably with the 676 base pairs of α1 interferon sequence present in pGW17 and pIF117. It is, therefore, difficult to explain why the readthrough products produced by pGW17 and pIF117, and pIF212, pIF213 and pIF217 terminate preferrentially at different sites in the vector (Figure 23). The α2 interferon readthrough species terminate at the more distal site.

Several of the species produced by the α2 producing plasmids seem too small to code for the mature protein. An 853 base pair probe derived from pIF211 was subjected to BglII digestion.Three fragments were obtained- 193 260 and 400 base pairs in length. The 193 and 260 base pair fragments

Figure 21: Analysis of total cell protein produced by repressed and induced α2 interferon constructions.

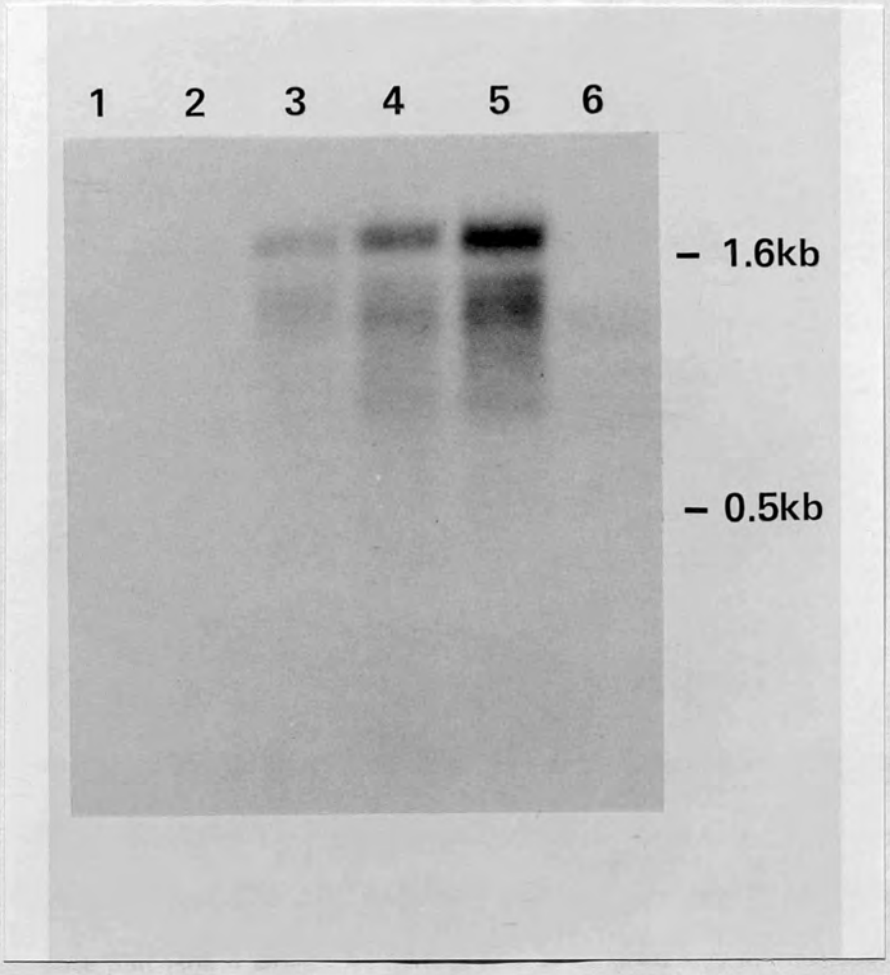


Total cell protein (20μg) was loaded onto a 12.5% polyacrylamide gel and electrophoresised and stained as described in General Methods 9.

Protein was isolated from cultures grown under both repressed (trp fed) and induced (trp starved and IAA addition) conditions.

Lane 1, protein standards. Lane 2, pBR322. Lane 3. pAT153. Lane 4, repressed pIF210. Lane 5, induced pIF210. Lane 6, repressed pIF211. Lane 7, induced pIF211. Lane 8. induced pIF117. Lane 9, repressed pIF212. Lane 10, induced pIF212. Lane 11, repressed pIF213. Lane 12 induced pIF213. Lane 13, repressed pWRL50. Lane 14, induced pWRL50.

Figure 22: Comparison of vector termination sites used by all the inteferon α2 constructions.

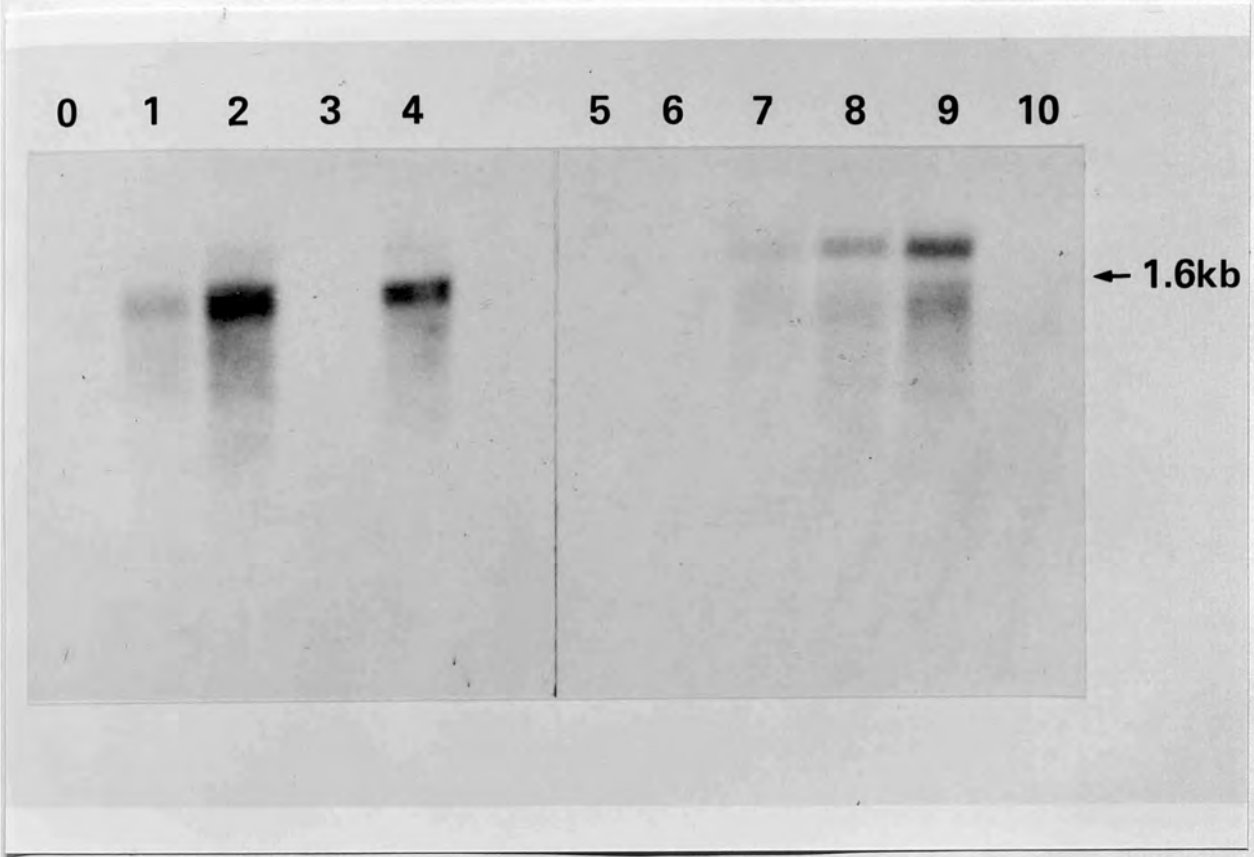


Total cellular RNA (8µg) isolated from all the interferon α2 constructions and pAT153 was probed with a nick translated 692 base pair AhaIII fragment prepared from pBR322 as described in Methods 3,(c), (ii).

Lane 1, pIF210. Lane 2, pIF211. Lane 3, pIF212. Lane 4, pIF213. Lane 5, pIF217. Lane 6, pAT153.

DNA size markers, 1.6 and 0.5 kilobases, are shown on the extreme right.

Figure 23: Comparison of vector termination sites utilised by interferon α1 and α2 constructions.



Total cellular RNA (8μg) isolated from each of the interferon α1 and α2 constructions, pBR322 and pAT153 was probed with a nick translated 692 base pair AhaIII DNA fragment prepared as described in Methods 3, (c), (i).

Lane 0, pBR322. Lane 1, pGW17. Lane 2, pIF117. Lane 3, pTH171. Lane 4, pTH172. Lane 5, pIF210. Lane 6, pIF211. Lane 7, pIF212. Lane 8, pIF213. Lane 9, pIF217. Lane 10, pAT153.

A DNA size marker, 1.6 kilobases, is shown on the extreme right.

contained regions of coding sequence while the 400 base pair fragment contained the very end of the coding sequence and all the non-coding region.

It has been shown that all the constructions produce the prematurely terminating mRNA species and several full length mRNA (Figure 19). However, transcription levels vary considerably among the constructions thus RNA from pIF217 (representative of α2 constructions) was probed with the fragments (193-8.8 x 10^6 dpm/μg, 260-3.0 x 10^7 dpm/μg, 400-2.0 x 10^7 dpm/μg). Figure 24 shows that there are indeed prematurely terminating mRNA produced by the α2 constructs. One mRNA species is recognised only by the 193 base pair fragment indicating that it terminates well within the coding sequence. Two other species are recognised by the 193 and 260 base pair probes revealing that they too are prematurely terminated transcription products. Thus three mRNA species produced by transcription of the α2 interferon sequence terminate within the coding sequence.

The interferon sequence was scanned by computer for any possible stable secondary structures that might occur. Two such structures were found within the coding region at bases 422 and 479 corresponding to the region in which the two species detected by the 193 and 260 base pair probes should terminate. (Figure 25.)

The computer predicted the formation of 5 hairpin loops, usually indicators of rho-independent terminators, in the pIF211 sequence. Construct pIF211 produces 6 interferon mRNA species. The only species that the computer did not predict a likely termination site for was the smallest species that terminates early in the coding region.

The remaining group of interferon mRNA species, those that are undetected by the AhaIII probe but are detected by the 400 base pair probe represent the largest class and are probably responsible for most of the α2 interferon that is produced. Constructs pIF211 and 213 produce similar quantities of these mRNA and produce similar quantities of interferon. While

Figure 24: Analysis of termination sites used by the interferon α2 constructions.

Total cellular RNA (8μg) isolated from pIF217 was probed with three nick translated fragments obtained by BglII digestion of the 853 base pair α2 sequence probe as described in Methods 3, (b), (ii).BglII cut the DNA at co-ordinates 288 and 548 (Goeddel et al. 1981) generating fragments 193, 260 and 400 base pairs in length as shown in panel (b).

Panel (a): Lane 1, 193 base pair DNA fragment. Lane 2, 260 base pair DNA fragment. Lane 3, 400 base pair DNA fragment.

DNA size markers,1.6 and 0.5 kilobases, are shown on the extreme right.

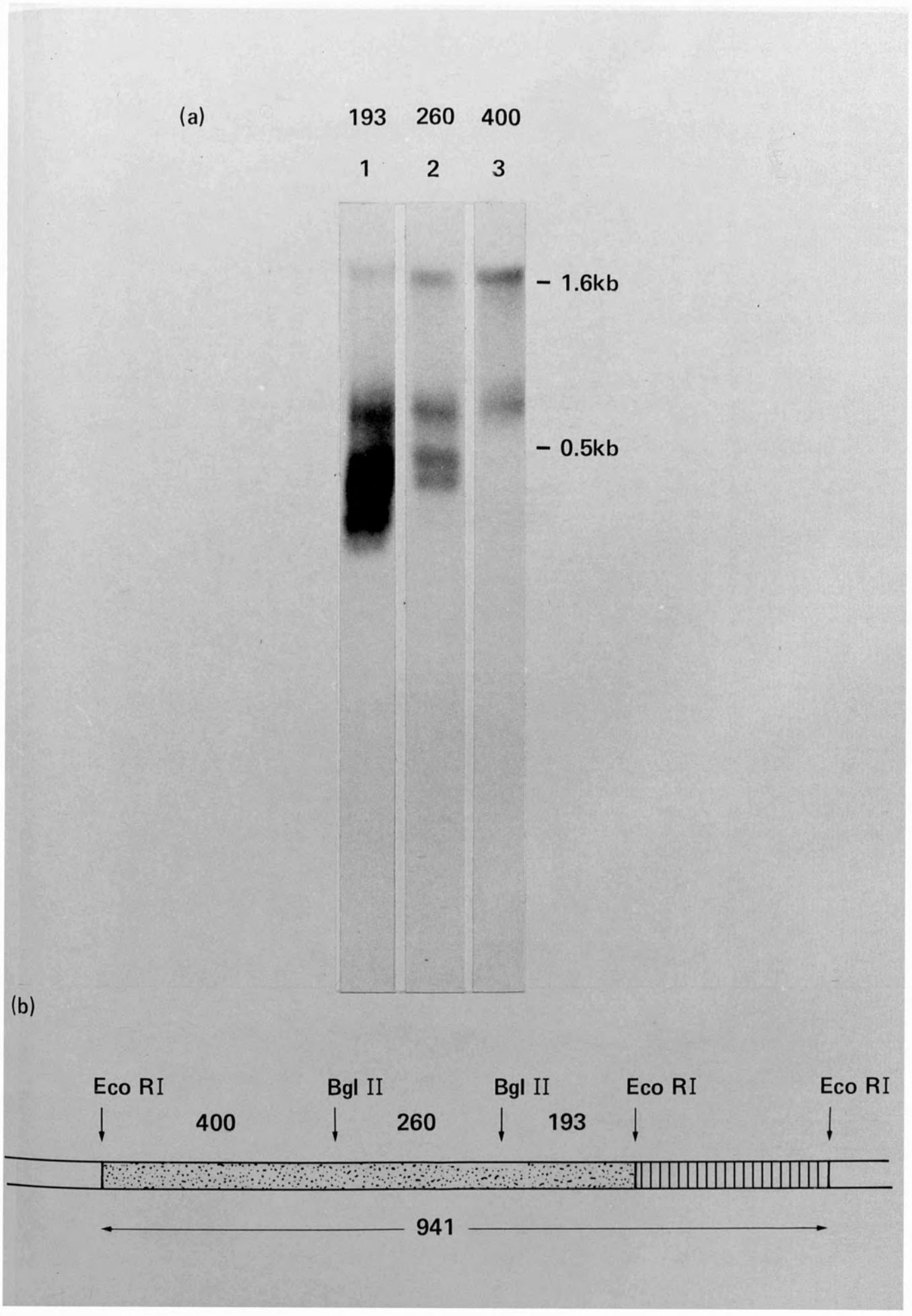
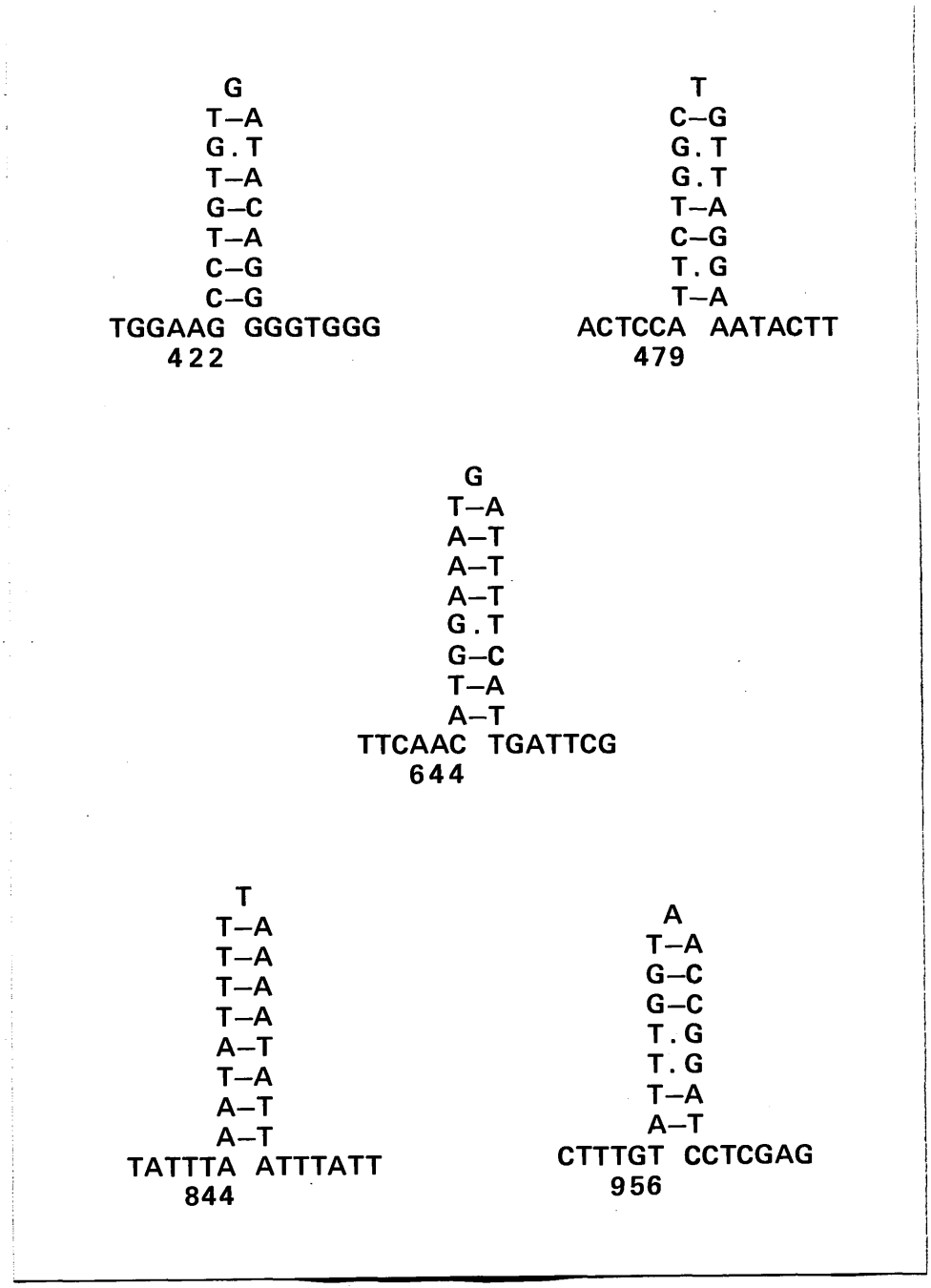

(a)
193
260
400
1
2
3
– 1.6kb
– 0.5kb
(b)
Eco RI
Bgl II
Bgl II
Eco RI
Eco RI
400
260
193
941

Figure 25: Potential stem-loop structures contained within the pIF211 DNA sequence.



Computer predicted stem-loop structures for pIF211 interferon DNA sequence. Coordinates, Goeddel et al. (1981), designate the commencement of the loops.

constructs pIF213 and pIF217 produce identical mRNA species pIF217 produces more readthrough mRNAs than does pIF213 (Figure 18). It is possible that the increased production of these species is responsible for the increased α2 interferon production. It may be that the readthrough species are more stable than the others or they may be more efficiently translated. In order to determine which mechanism was responsible for the increased interferon production mRNA half life studies were undertaken.

5. Half-lives of interferon α2 mRNA species:

Rifampicin(0.2mg/ml)was added to exponentially growing cultures blocking further transcription initiation by RNA polymerase. At selected time intervals samples were removed and the RNA purified. The samples were then hybridised to the 853 base pair α2 interferon specific probe (2.1 x 10^8 dpm/μg). The results obtained are shown in Figure 26.

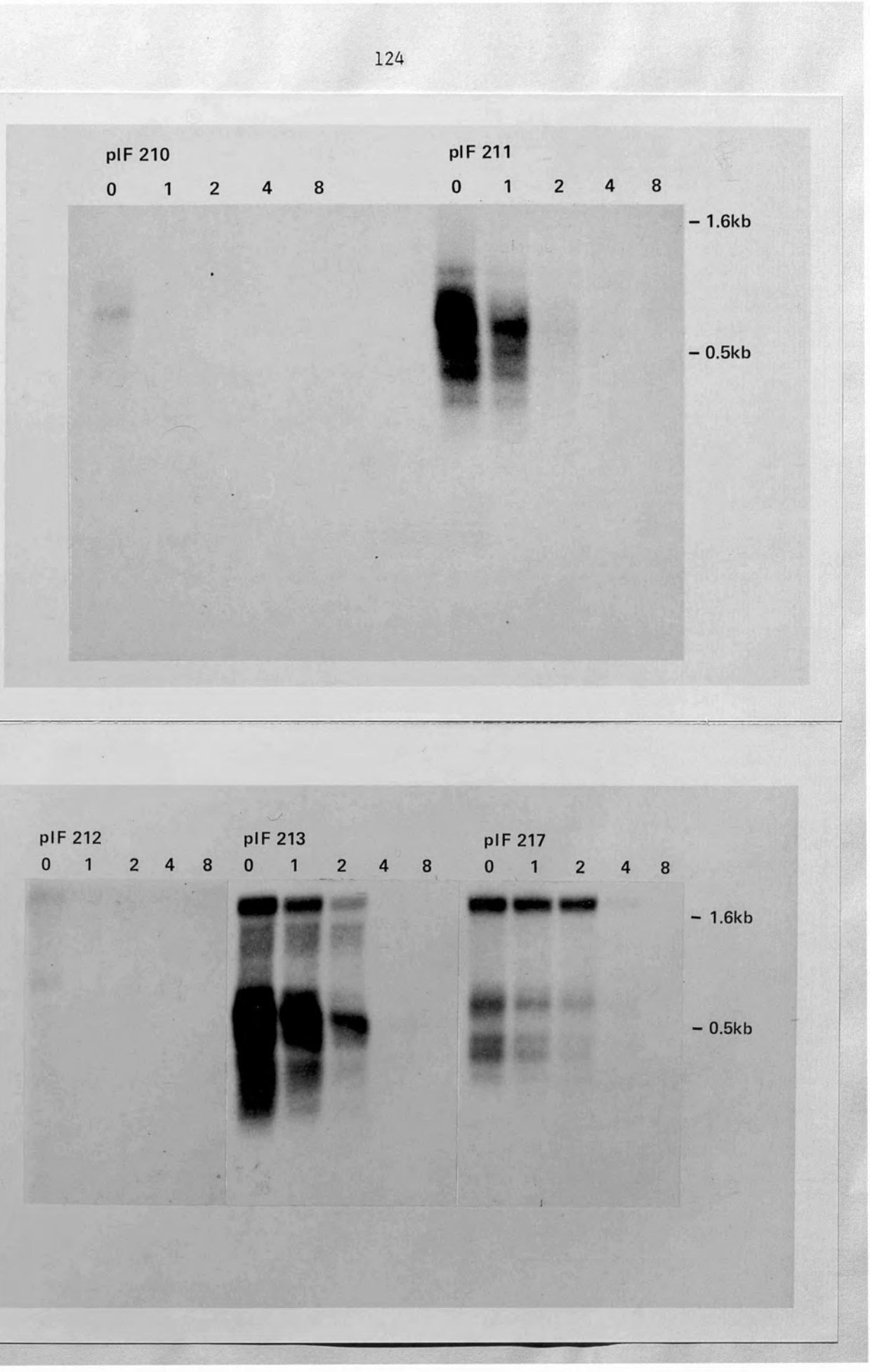
The single detectable species produced by pIF210 decayed with a half-life of less than one minute. The multiple species produced by pIF211 decayed uniformly with a half-life of approximately 1.0 minute, as did the mRNAs produced by pIF212 and 213. The mRNAs produced by pIF217 appeared slightly more stable. The half-lives of the smaller species were approximately 1-1.5 minutes, just fractionally longer than the identical mRNA species produced by the other constructs. The readthrough mRNA species behaved quite differently. The mRNAs appeared completely stable for the first 2 minutes and then very rapidly disappeared, being barely detectable at 4 minutes. It is possible to interpret this result in two ways. Firstly, that the half-lives of these species are approximately 3 minutes. Alternatively, the rapid degradation after 2 minutes suggests that the half-lives are similar to those of the other interferon mRNAs and are only 1-1.5 minutes. For this to be so the messages must be protected in some way from degradation. It has been proposed that ribosomes actively translating a message may help protect it from degradation. pIF213 and pIF217 have been

Figure 26: Comparison of interferon α2 mRNA half-life profiles of all the constructions.

Total cellular RNA (8μg) isolated from cultures at 0, 1, 2, 4 and 8 minutes after rifampicin treatment was probed with a nick translated 853 base pair interferon α2 DNA fragment prepared as described in Methods 3, (b), (i).

The half-lives of the mRNA species produced by constructions pIF210, pIF211, pIF212, pIF213 and pIF217 were all determined.

DNA size markers, 1.6 and 0.5 kilobases, are shown to the right of pIF211 and pIF217.


pIF 210
0 1 2 4 8
pIF 211
0 1 2 4 8
– 1.6kb
– 0.5kb
pIF 212
0 1 2 4 8
pIF 213
0 1 2 4 8
pIF 217
0 1 2 4 8
– 1.6kb
– 0.5kb

shown to produce identical mRNA species. The delay in onset of degradation of the readthrough product is seen with pIF217 and not with pIF213.

A combination of factors, including increased mRNA stability and increased translational efficiency are responsible for the high levels of α2 interferon obtained from pIF217. As good as they may be in comparison with the other constructs developed the absolute levels of α2 interferon produced by pIF217 are poor when compared with α1 production.

6. Summary of results:

A series of plasmids containing the α2 interferon sequence but differing in selected regions of non-coding sequence were developed in an attempt to obtain a construct capable of producing significant amounts of interferon. The best α2 producer, pIF217, was able to produce similar levels of interferon per plasmid copy as that obtained from the best α1 constructs. However, in absolute terms α2 production never reached the same level as α1 production (pTH172 13mg/l, pIF217 4mg/l). The α1 sequence increased the plasmid copy number of the vector into which it was inserted but no such affect was obtained with the α2 sequence. Plasmid copy numbers of all the α2 constructs were similar to the vector in which they were inserted and while altering selected regions of the non-coding sequence affected protein production copy number was unaffected.

Another difference between the plasmids containing the two interferon sequences was their stabilities. All the α1 containing plasmids were 100% stable over 40 generations. Although pIF217 was 100% stably maintained, all of the other α2 containing plasmids were unstable to a greater or lesser extent under the conditions tested. The degree of instability increased with increased interferon production. Most of the α2 plasmids were pAT153 based except pIF217, which contained the α2 sequence on the lower copy number vector pBR322.

Analysis of the transcription products of the constructs provided a simple explanation for the poor interferon production by some of the constructs. Not completely unexpected in view of the α1 results was the production of multiple mRNA species, but a comparison of the quantities of mRNA produced revealed that two, pIF210 and pIF212, produced significantly less interferon message than any of the others. These two constructions contain a deletion 5' to the -35 region of the trp promoter and it appeared that this region was important for promoter function. Perhaps it aids in recognition of, or entry to the promoter site.

Like pGW17 and pIF117 multiple messages were produced by all the α2 interferon constructs. In those that had had the non-coding sequence shortened to a similar length as pGW17 and pIF117 long readthrough mRNA species were detected. Where the non-coding sequence had been left intact termination occurred efficiently within this region and no mRNA containing vector sequence were detected. In the constructs that produced long mRNA species, interferon α2 and α1 production was accompanied by a concomitant accumulation of β-lactamase, detected by electrophoresis of total cell protein on polyacrylamide gels. This provided the first indication that the long messages may be polycistronic. Detection of these long species by both the interferon specific probes and the AhaIII fragment supported this assertion. Suprisingly, the α1 and α2 polycistronic messages favoured different pBR322/pAT153 termination sites. The α1 containing readthrough mRNAs preferrentially terminated at sites further upstream than the α2 readthrough messages. There is no obvious reason to explain why the preferred termination sites should vary between α1 and α2 interferon producers.

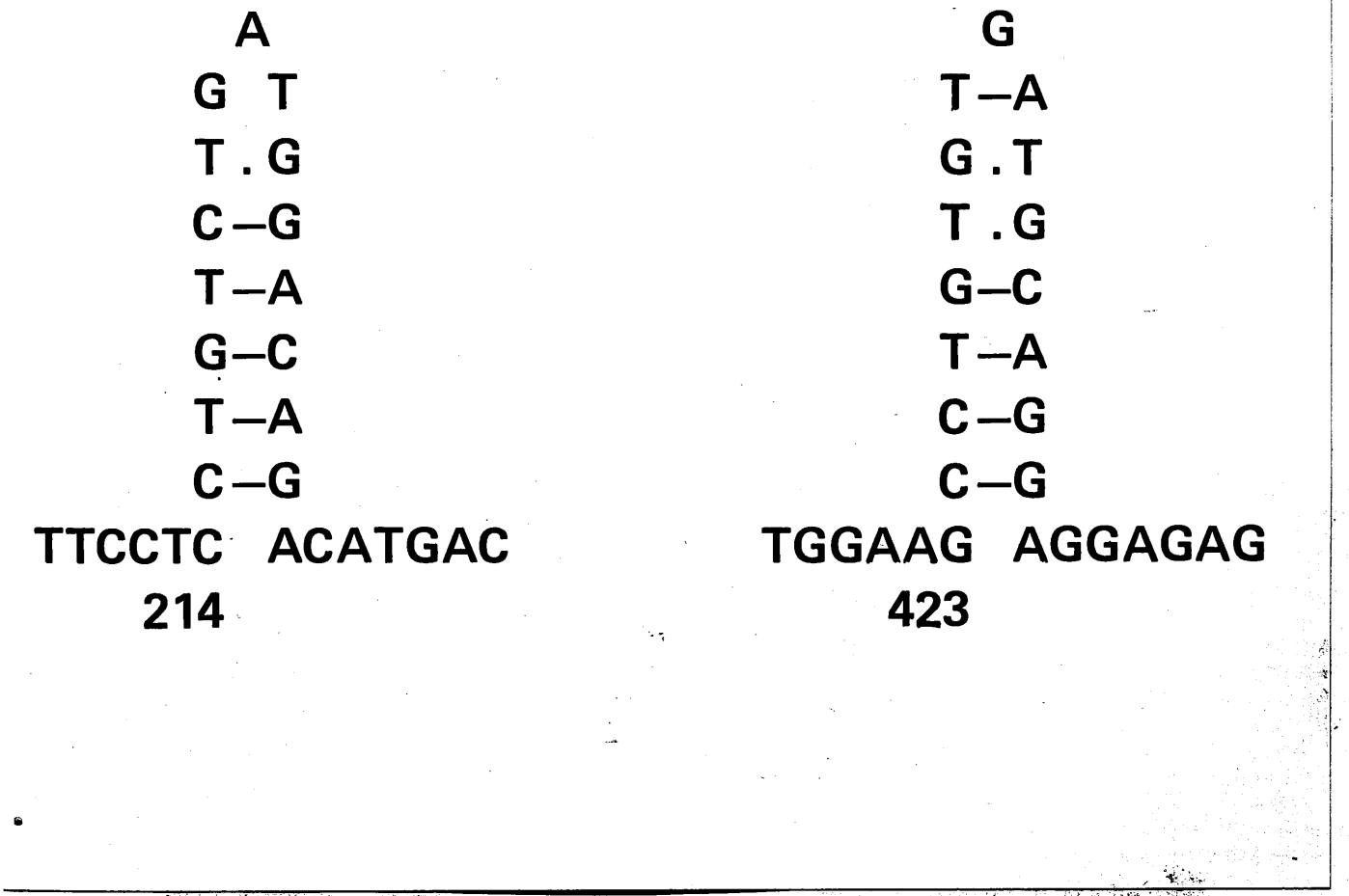
Among the many mRNA species produced by the α2 interferon constructions there were several that appeared too small to contain all the information required for translation into mature α2 interferon. Indeed three

small mRNA species were shown to terminate prematurely within the coding sequence. In contrast to α1, transcription of the α1 sequence produced no prematurely terminated mRNA species. Computer analysis of the α2 sequence revealed two positions within the coding region where hairpin loops might form. Hairpin loop formation is usually associated with rho-independent termination, or transcription pausing. The areas in which the computer predicted stable hairpin loop formation within the coding sequence coincided with the region shown by the BglII fragments to contain two termination sites. The computer did not predict a termination site for the smallest mRNA species. Computer analysis of the α1 interferon sequence produced two potential stem-loop structures at positions 214 and 423 base pairs (Figure 27). The first, had no α2 equivalent, while the second was very similar to the structure present at 422 in the α2 sequence. Stem-loop structures do not always give rise to termination and no α1 mRNA species of appropriate size have been detected.

The production of prematurely terminating mRNA species by E. coli underlines a problem encountered when expression of eucaryotic proteins is undertaken in procaryotic systems. E.coli apparently recognises signals within the sequence that direct termination of the RNA polymerase activity. Presumably termination does not occur at these sites when the gene is transcribed in leucocytes.

The half-life studies revealed one very interesting point- the abnormal decay pattern of the readthrough products produced by pF217. These mRNAs appear resistant to degradation for the first 2 minutes, then they decay rapidly, being hardly detectable at 4 minutes after rifampicin addition. None of the other mRNAs exhibited this degradation profile. Even the readthrough products produced by pIF212 and pIF213 which, except for three bases in the ribosome binding site region, are identical to those produced by pIF217, decay uniformly with half-lives of 1-1.5 minutes. The changes to the ribosome

Figure 27: Potential stem-loop structures contained within the pIF117 DNA sequence.



Computer predicted stemloop structures within the pIF117 sequence. Co-ordinates designate the commencement of the loop.

binding site involve the deletion of two bases and because this produces a ribosome binding site that conforms more closely to the consensus site this should increase translation (Gold et al. 1981). The apparent resistance to degradation is not observed for all mRNAs produced by pIF217, only the readthrough species exhibit this behaviour. If increased translation was the sole factor responsible for the delayed onset of degradation then all interferon containing messages should behave similarly. Von Gabain et al. (1983) observed the same delayed onset of degradation for the pBR322 encoded β-lactamase gene. The pIF217 readthrough messsages contain the β-lactamase sequence. However, no delayed onset of decay was observed for the α1 readthrough mRNA species whioh also contain this region. The plasmids that produce the α1 readthrough mRNA contain the same ribosome binding region as pIF217 and translation is expected to be as efficient in these constructs. It has long been argued that the presence of translating ribosomes on a message could protect it from degradation. Although such a mechanism must be postulated in this case it is hard to explain why these same ribosomes offer no protection to the interferon mRNA or to the read through products obtained from the α1 interferon producers.

Comparison of mRNA profiles of pIF213 and pIF217 revealed that although the species produced are identical they are produced in different proportions by the two constructions. More readthrough mRNAs are produced proportionately by pIF217. While these species are arguably more stable the increased stability alone cannot explain the 8 fold increase in interferon production. It is difficult to explain why pIF217 produces more readthrough mRNA than pIF213.

It is apparent from these experiments that although manipulation of DNA sequences can give rise to increased protein production, expression of foreign proteins may be limited by sequence content in some systems.

C. pIF217 mRNA half-life determinations and interferon production in different media and at differing growth phases:

Nilsson et al. (1984) have shown that some bacterial gene products respond to changes in growth rate by altering the stabilities of their mRNA products. The growth-related affects were observed for both very stable mRNA and those decaying with a half-life of 2-3 minutes typical of most E.coli mRNAs. Their findings showed that certain E.coli messages are degraded more rapidly in media supporting slow growth. Investigations into the stabilities of pIF217 mRNAs were undertaken to determine whether any of the species, particularly the readthrough message, were subject to changes in stability at differing cell growth rates.

1 Half-lives of interferon mRNA species at differing growth phases:

RNA was isolated from cultures growing under maximally induced conditions in MOPs glucose medium at three different growth stages. These different growth stages, lag, exponential and early stationary phase corresponded to differing growth rates for the plasmid.

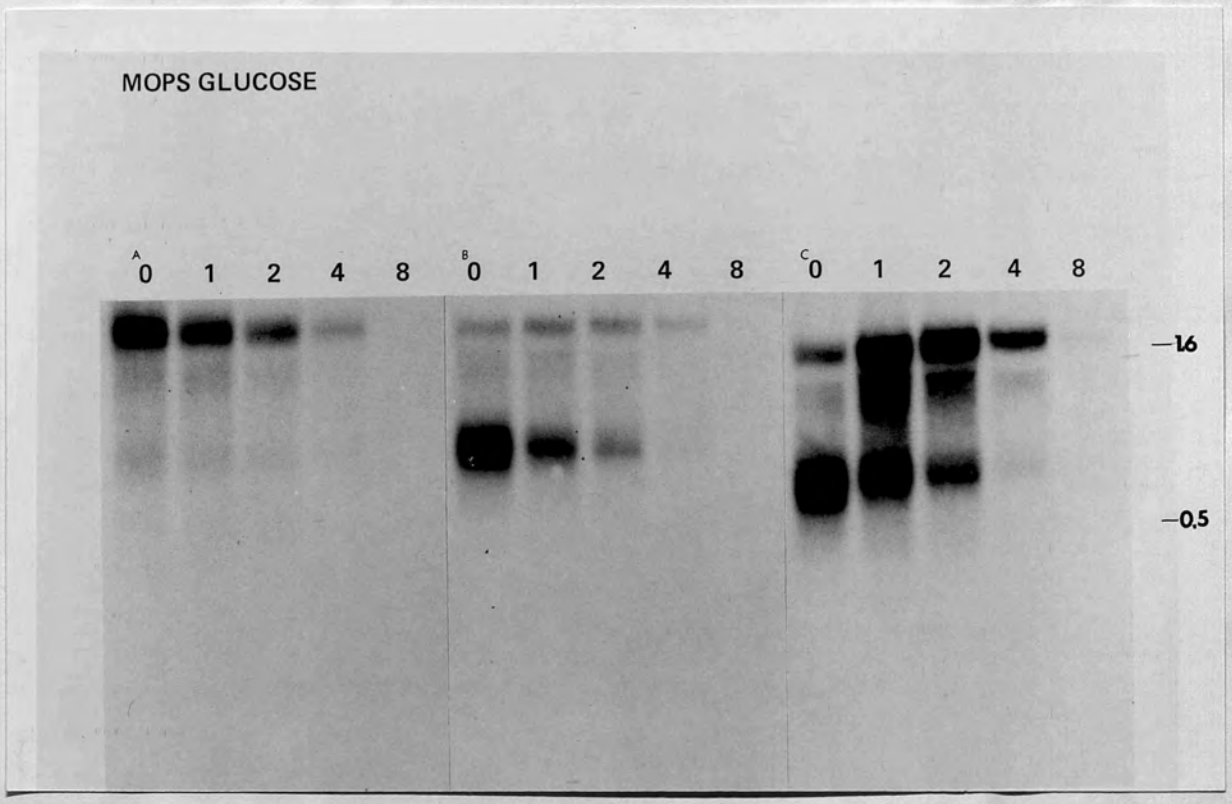
Rifampicin (0.2mg/ml) was added to the growing culture to prevent further transcription initiation by RNA polymerase. Samples were withdrawn from the growing cultures at selected time intervals and total RNA was purified. The RNA was then glyoxalated, Northern bbtted onto Pall biodyne and hybridised to an $\alpha 2$ specific nick translated probe (4×10^7 dpm/µg). The results are shown in Figure 28.

The mRNA profiles obtained are very different. Although all the mRNA species are produced at all phases of growth their relative abundances differ. In the lag phase the long readthrough messages are predominant and only a little of the smaller mRNAs are produced. In contrast, cultures growing exponentially and those entering stationary phase favour the production of the smaller mRNA species. All the mRNAs produced in the lag phase decay uniformly with a half-life of 1-1.5 minutes. Similarly, the small mRNA

Figure 28: Comparison of the stabilities of interferon mRNAs produced by construct pIF217 at three different growth phases in MOPs glucose medium.

Total cellular RNA (8µg) was isolated at 0, 1, 2, 4, and 8 minutes after rifampicin treatment of lag, exponential and stationary phase cultures containing plasmid pIF217. The RNA was probed with a nick translated 853 base pair DNA fragment prepared as described in Methods 3, (b), (i).
Panel (a) shows the decay of RNA isolated from lag phase cultures. Panel (b) shows the decay of RNA isolated from exponential phase cultures. Panel (c) shows the decay of RNA isolated from stationary phase cultures.

DNA size markers, 1.6 and 0.5 kilobases, are shown on the extreme right


MOPS GLUCOSE
A 0 1 2 4 8
B 0 1 2 4 8
C 0 1 2 4 8
1.6
0.5

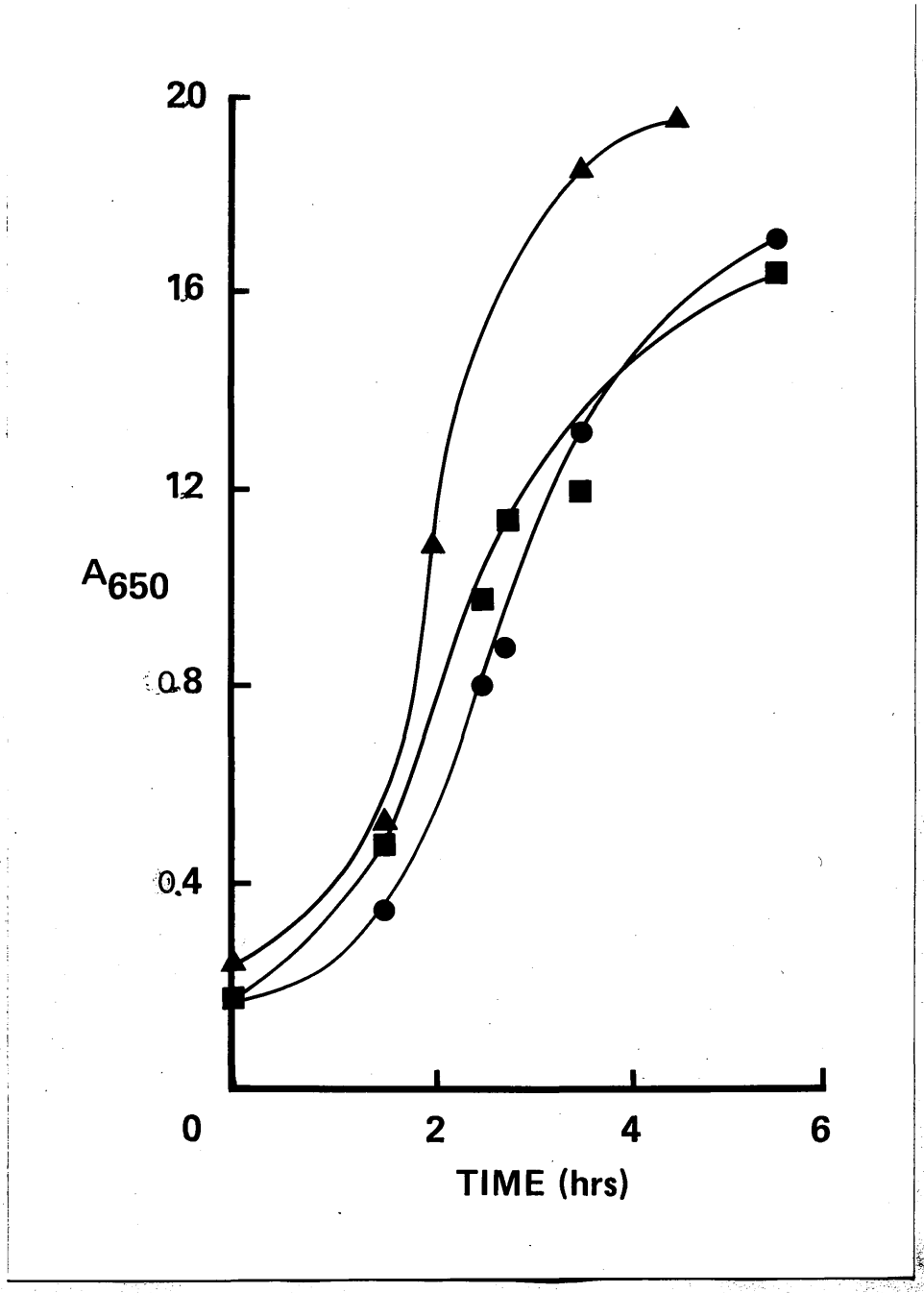
produced at the other two growth stages decay uniformly with a half life of 1-1.5 minutes. This is not so for the readthrough mRNA produced in exponentially growing cultures and those just entering stationary phase. The behaviour of these species is quite unexpected. There is an increase in readthrough mRNAs at 1 minute after rifampicin addition, which, in the exponentially growing cultures, remains unaltered until T=2 and then decays with a half-life of 2 minutes. However, the same species in the early stationary phase continue to increase in abundance until 2 minutes after rifampicin addition and then decay with an apparent half-life of 2 minutes. This increase in adundance of the readthrough mRNAs is not observed in lag phase cultures.

It appeared that the interferon mRNAs were responding to changes in cell growth rate but in a quite unexpected and not easily explainable way. To confirm that this was simply a growth rate response the half-lives of the interferon mRNA species and relative abundances were determined in different media that corresponded to different growth rates. Included in this study were two media with identical growth rates but differing in composition to see whether medium per se had any affect on mRNA stability or relative mRNA species abundance.

2. A comparison of the half-lives obtained from plasmid pIF217 grown in different media:

The growth rate of the interferon producing construct pIF217 was determined in each of the media used. Figure 29 shows that polypeptone glucose supported the fastest rate of growth with the cells doubling every 40 minutes while MOPs glucose and M9CA allowed the cells to double every 60 minutes. A comparison of the half-lives of the interferon mRNAs produced in these media at exponential growth phase shows not only an affect of cell growth rate on mRNA stability but also an affect of media on mRNA stability. Again, rifampicin was used to arrest transcription initiation by RNA

Figure 29: Comparison of growth rates of construct pIF217 in polypeptone glucose, M9CA and MOPs glucose media.



Cultures (25ml) induced by trp starvation and IAA addition were grown at 37°C in an orbital shaker as described in General Methods 1. Samples (0.5ml) were removed at appropriate time intervals after IAA addition and the absorbance at 650nm determined.

Polypeptone glucose (△). M9CA (○). MOPs glucose (□).

polymerase and total RNA was purified from samples removed at appropriate times. The interferon mRNAs were detected as previously described and the results are shown in Figure 30.

In all the media both mono- and polycistronic interferon messages are produced in approximately the same proportions. The small, prematurely terminating species are present in all media and, as seen earlier (Figure 28), at all growth phases they represent a very minor component of the total interferon mRNA species.

The readthrough mRNA produced in polypeptone glucose medium although apparently unaltered in amount at T=1, show no increase in abundance unlike the same mRNA species produced in the other two media. The apparent decrease in stability of these mRNA species at this increased growth rate, is contrary to the findings of Nilsson et al. (1984.)

The half-lives of the intermediate mRNA species are similar in all three media, 1-1.5 minutes. It is difficult to determine the half-lives of the readthrough mRNAs produced in MOPs glucose and M9CA media because of their changing stabilities. The half-lives of the polypeptone readthrough mRNA are 1-1.5 minutes, the same as those obtained for the intermediate species.

3. Interferon production in different media:

Experiments were designed to see if there was a relationship between the mRNA stabilities obtained in the different media and levels of interferon produced. Interferon production was determined by bioassay of S100 lysates prepared from the exponentially growing cultures harvested at the same time as that used in the stability studies.

Figure 30: Comparison of the stabilities of mRNA species produced by construct pIF217 in polypeptone glucose, M9CA and MOPs glucose media.

Total cellular RNA (8µg) was isolated at 0, 1, 2, 4 and 8 minutes after rifampicin treatment of cultures containing plasmid pIF217 grown in polypeptone glucose, M9CA and MOPs glucose media. The RNA was probed with a nick translated 853 base pair DNA fragment (1.32×10^7 dpm/µg) prepared as described in Methods 3, (b), (i).

DNA size markers, 1.6 and 0.5 kilobases, are shown on the extreme right.

Table 7 shows that maximum interferon production was obtained from cultures grown in polypeptone glucose medium. M9CA and MOPs glucose media produced similar amounts but only half of that produced in polypeptone glucose medium. The levels of interferon produced by pIF217 in this set of experiments vary from those previously determined (Table 5).Although the method of interferon quantitation produces variations in absolute levels between successive determinations the values are consistent within the particular experiment.

If mRNA stabilities influenced interferon production directly one might predict that interferon production would be greatest in M9CA and MOPs glucose media where production of the readthrough mRNA species that is apparently initially resistant to degradation occurs. However, this is not so. Production of the mRNA species in MOPs glucose and M9CA media that are apparently resistant to degradation do, however, give rise to greater β-lactamase production as shown on polyacrylamide gels (Figure 31).

As found earlier (Tables 4 and 5) plasmids containing the α2 interferon sequence are unstable and the degree of instability correlates with the level of interferon produced and the ability of the construct to express tetracycline resistance. Those constructions containing the intact tetracycline gene are slower growing in liquid culture than constructions lacking this sequence (Figure 32). pIF217 contains the genes for ampicillin and tetracycline resistance and therefore may be selected for by the inclusion of either of these antibiotics in the growth medium. Table 7 shows that the choice of antibiotic marker had little affect on the quantities of interferon produced. The use of tetracycline as the selection antibiotic had, however, a profound affect on growth of cultures in polypeptone glucose and MOPs glucose media. In both cases the cultures entered a very long lag phase of 3-4 hours. Once growth resumed the cells doubled at the previously determined rate.

Table 7: Quantitative analysis of interferon production by construction pIF217, with and without tetracycline, in polypeptone glucose, M9CA and MOPs glucose media.

Media	Amount of interferon produced (mg/l) of culture	
	- tetracycline	+ tetracycline
polypeptone glucose	0.48	0.48
M9CA	0.12	0.25
MOPs glucose	0.18	0.25

The data presented here represents the average of four separate determinations.

Interferon protein levels were determined by bioassay of S100 lysates (General Methods 12).

Figure 31: Comparison of total cell protein produced by construct pIF217 in polypeptone glucose, M9CA and MOPs glucose media.

Total cell protein (20µg) prepared from IAA induced construct pIF217 grown in polypeptone glucose, M9CA and MOPs glucose media was loaded onto a 12.5% polyacrylamide gel and electrophoresised and stained as described in General Methods 9.

Total cell protein was isolated 0, 1, 2, 4 and 8 minutes after cells were treated with rifampicin to prevent further transcription initiation by RNA polymerase.

Lane 1, protein standards. Lane 2-5 contain protein isolated from cultures grown in polypeptone glucose medium. Lanes 6-9 contain protein isolated from cultures grown in M9CA medium. Lane 10-13 contain protein isolated from cultures grown in MOPs glucose medium.

The arrow indicates the position of the β-lactamase protein.

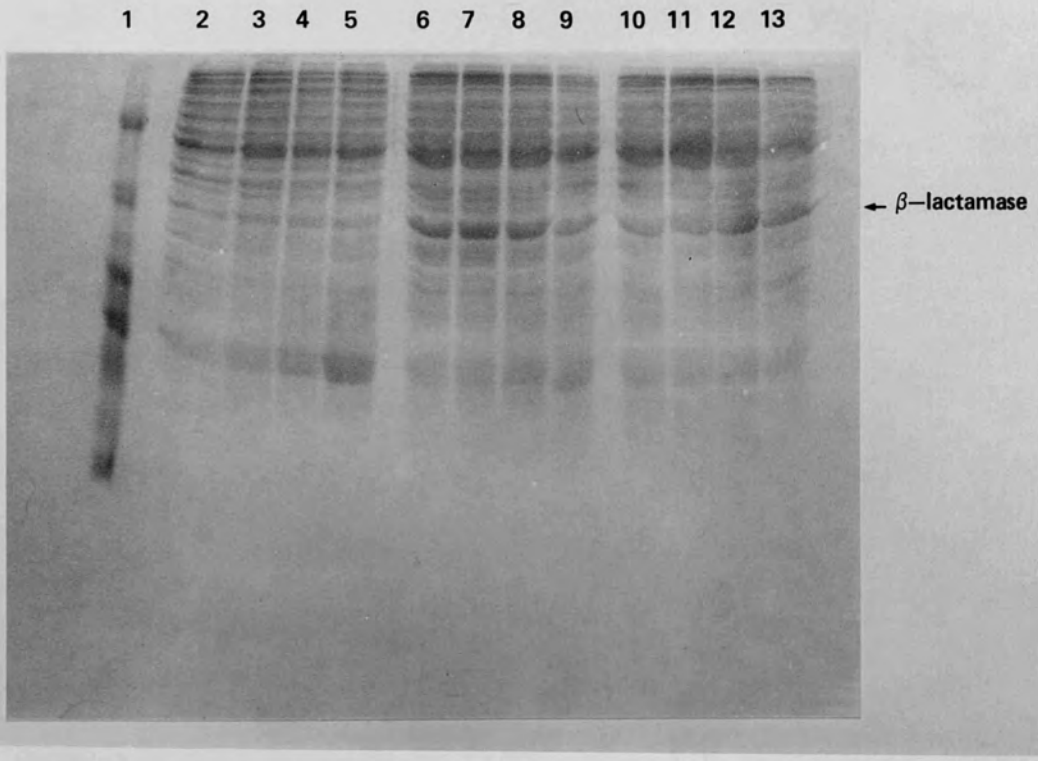
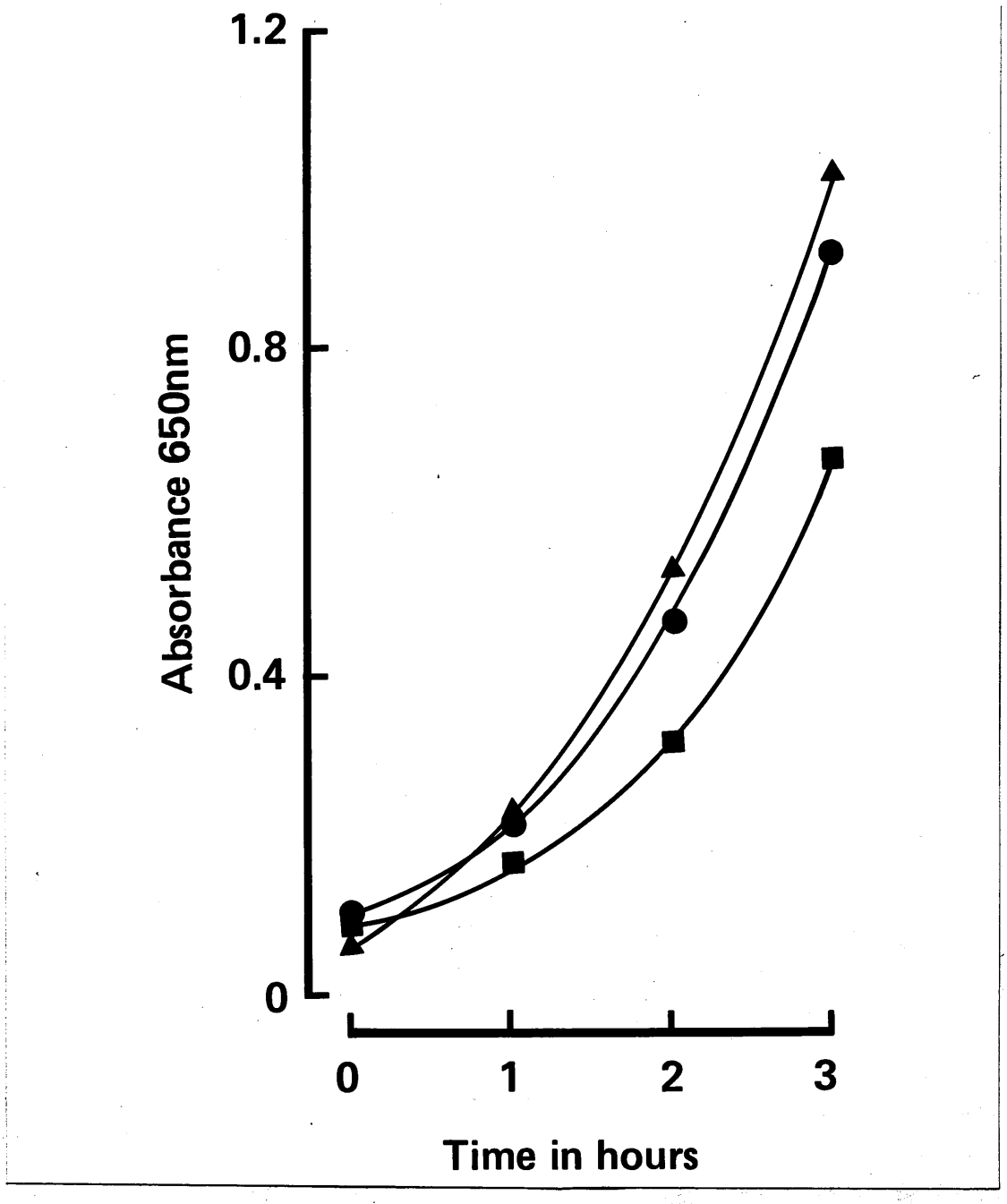

1 2 3 4 5 6 7 8 9 10 11 12 13
β—lactamase

Figure 32: Comparison of growth rates of constructs pIF210, pIF212 and pIF217.



Cultures (25ml) induced by IAA addition and trp starvation were grown at 37°C in an orbital shaker as described in General Methods 1. Samples (0.5ml) were removed at 0, 1, 2 and 3 hours after IAA addition and the absorbance at 650nm determined.

pIF210 (▲). pIF212 (●). pIF217 (■).

There seems to be no clear relationship between mRNA species stability and interferon production in the media studied. Interferon production appears to increase in response to increased growth rate.

4. Summary of results:

The results reported here show that certain mRNAs are more stable in media supporting slow growth. These results are in contrast to those obtained by Nilsson et al. (1984) suggesting that there is no general mechanism for determining changes in mRNA stability in E.coli such as the levels of RNA degrading enzymes.

Both mono and polycistronic mRNAs are produced under all the experimental conditions but the proportion of one type to another and the relative stabilities of the two types varied depending on the culture conditions employed.

When the half-lives of the interferon mRNA were determined at varying growth phases in MOPs glucose medium some suprising differences were observed. The predominant mRNA species produced in lag phase were the readthrough products and only small quantities of the other mRNA species were detected. At the other two growth phases production of the smaller mRNAs were favoured. It is obvious that in MOPs glucose medium the cells in lag phase favour a single transcriptional event producing polycistronic messages coding for interferon and β-lactamase. The production of polycistronic messages requires that the polymerase transcribes regions of DNA producing mRNA that are not translated.It may be that the synthesis of polycistronic mRNAs is energetically less demanding than the promotion and termination of multiple monocistronic mRNAs and therefore would be favoured in cells grown in minimal (MOPs glucose) rather than nutritious (polypeptone glucose) media.

All the mRNAs produced in the MOPs glucose lag phase decay uniformly with half-lives of 1-1.5 minutes. The mRNAs isolated from cultures growing in MOPs glucose at exponential or early stationary phases exhibited a much more complex degradation pattern. The smaller mRNAs decayed uniformly with half-lives of 1-1.5 minutes but the readthrough products appeared to increase in abundance until two minutes after rifampicin addition. They then decayed uniformly with half-lives of 2 minutes. The increase in the readthrough mRNAs was more pronounced in the later phase of growth. Cells grown on M9CA medium showed a similar pattern of mRNA decay as that observed for the exponentially growing MOPs glucose culture. The small mRNAs decayed uniformly with half-lives of 1-1.5 minutes. There was an apparent increase in abundance of the readthrough products until two minutes after rifampicin addition when the mRNAs decayed uniformly with half-lives of 2 minutes. Interferon mRNA species isloated from cultures grown in polypeptone glucose medium did not display the same behaviour. While the readthrough products did not decay appreciably in the first minute after rifampicin addition they did not increase in abundance. All the mRNA species produced by pIF217 grown in polypeptone media decayed with the same half-life-1-1.5 minutes.

Delayed onset of decay of mRNA has been reported by von Gabain et al. (1985). They observed that pBR322 β-lactamase mRNAs were resistant to degradation until 2 minutes after rifampicin addition. At this time decay commenced and was uniform. They claimed that the delayed onset in decay was due to the completion of nascent transcripts. Rifampicin bound to free RNA polymerase molecules and rendered them incapable of further transcriptional initiation whilst those RNA polymerase molecules already engaged in active transcription were unaffected.

In procaryotic systems where transcription and translation are considered to be coupled it is possible that ribosome presence and not the

polymerase molecule is responsible for the apparent delay in onset of decay. It may be that translation by ribosomes is the rate determining and thus the apparent resistance determining factor. Pedersen (1984) reported on the variable in vivo translation rates for different mRNAs and showed that translation of β-lactamase mRNA was about 50% slower than expected from the translation time of the ribosomal proteins measured in the same experiment. This correlated well with the high content of rare codons in the β-lactamase gene. When pIF217 cultures are grown in minimal media such as MOPs glucose and M9CA the effects of codon composition on translation rates could be expected to be greater than in cultures grown in more nutritious medium because more cellular requirements have to be synthesised de novo in minimal media. The mRNA stabilities of the readthrough products obtained in the three different media reflect the proposed variations in translation rates. When the rare codon content of the α2 interferon sequence was calculated using the same criteria as that applied by Pedersen (1984) the rare codons occurred with a frequency of 27%. Slow translation of α2 interferon mRNA might therefore be expected. However, only the readthrough mRNA produced by construct pIF217 demonstrated this delay in the onset of degradation. Differences in translational efficiency may explain the inability of the other α2 polycistronic messages produced by constructs pIF212 and pIF213 to appear resistant to degradation. The use of upstream termination sites by the α1 readthrough species may explain their uniform degradation pattern. It is possible that a combination of factors, including DNA sequences at the termination site and slowed elongation rates, results in the apparent stability of the readthrough mRNA species produced when pIF217 is grown in minimal media.

Interferon production is maximal in cultures grown in polypeptone glucose medium.It appears that production of interferon increases with increasing growth rate. Regulation of protein production must occur at the translational and not transcriptional level.

D. Identification of an α2 interferon mRNA species terminated at a rho-dependent site.

Multiple interferon mRNA species were produced by pGW17, pIF117 and all the α2 interferon constructions. It has been shown that three mRNA produced by transcription of the α2 sequence terminate within the coding sequence while transcription of the α1 sequence produces only full length messages. Computer analysis of the α2 sequence data produced two potential stem-loop structures in positions in the coding sequence that coincided with the termination sites for two of the observed mRNAs. A possible termination site was not predicted for the third and smallest mRNA species. Since stem-loop structures are usually associated with species termination at rho-independent sites it is possible that the remaining site is subject to rho-dependent termination.

Two isogenic E.coli strains HD152 and KH54 were obtained from the E.coli Stock Centre. HD152 produces a temperature sensitive rho protein and growth of the culture could not be sustained at 42°C. KH54 produces a wild type rho protein and is capable of continued growth at 30°C and 42°C.(Inoko et al. 1977.)

Comparison of interferon α2 mRNA produced in these two strains should help to determine whether or not any of the mRNAs are terminated at rho-dependent sites in vivo.

1. Construction of rho- trp+ and rho+ trp+ strains:

The two isogenic E.coli strains obtained from the E.coli Stock Centre contained an amber mutation that necessitated the addition of tryptophan to the medium to enable their growth. It was essential to obtain trp revertants because the interferon gene is promoted from the trp promoter which is subject to control by tryptophan. If tryptophan must be supplied to the cultures in order for them to grow interferon production would be continually repressed.

The two strains were plated out on to M9CA plates containing no tryptophan to select natural revertants. Colonies that grew after an overnight incubation at 30°C were trp+. Their growth response to a temperature shift from 30°C to 42°C was assessed (Figure 33) to ensure that no alteration to the rho phenotype had occurred. Both strains required supplements of several other amino acids (Appendix A). HD152 trp+ and KH54 trp+ were unaltered in their amino acid requirements expect with respect to tryptophan. (Table 8).

2. Transformation of the plasmids into rho+ trp+ and rho- trp+ strains:

Plasmids pIF117, pTH172 and all the α2 interferon producing plasmids were transformed into HD152 trp+ and KH54 trp+. The α1 and all the α2 plasmids except pIF217 transformed readily into both strains and were selected for by growth on L-broth plates containing ampicillin (40μg/ml). Very few colonies were obtained when pIF217 was transformed into HD152 trp+ and incubated at 30°C overnight. When those colonies were grown in liquid culture and plasmid DNA prepared, no plasmid DNA could be detected. The plasmid could be maintained in culture with tetracycline (12.5μg/ml) but cultures grown in the presence of tetracycline grew very slowly.

Plasmid DNA was prepared from all the newly formed strains (HD210, HD211, HD212, HD213, HD117, HD172, KH210, KH211, KH212, KH213, KH117, KH172- see Appendix A) and linearised by Pst 1 digestion. The plasmid inserts were sized by comparison with DNA standards after electrophoresis on a TBE 1% agarose gel (Figure 34).

Varying constructs produced low quantities of interferon mRNA because of plasmid instability (HD213 and HD217) or low transcription levels (HD210 and HD212). Plasmid pTH172 contains the threonine attenuator, a known rho-independent terminator which in the presence of threonine promotes termination of the mRNA species produced by strains containing

Figure 33: The effects of temperature on the growth of a rho temperature sensitive strain and an isogenic strain containing a wild type rho protein in polypeptone glucose medium.

HD152 trp+ (o) and KH54 trp+ (□) cultures growing exponentially at 30°C were divided in half. One half was kept at 30°C (A and C) while the other half was transferred to 42°C for further incubation (B and D). Cell growth was measured by optical density at 650nm (A and B) and by colony formation at 30°C (C and D).

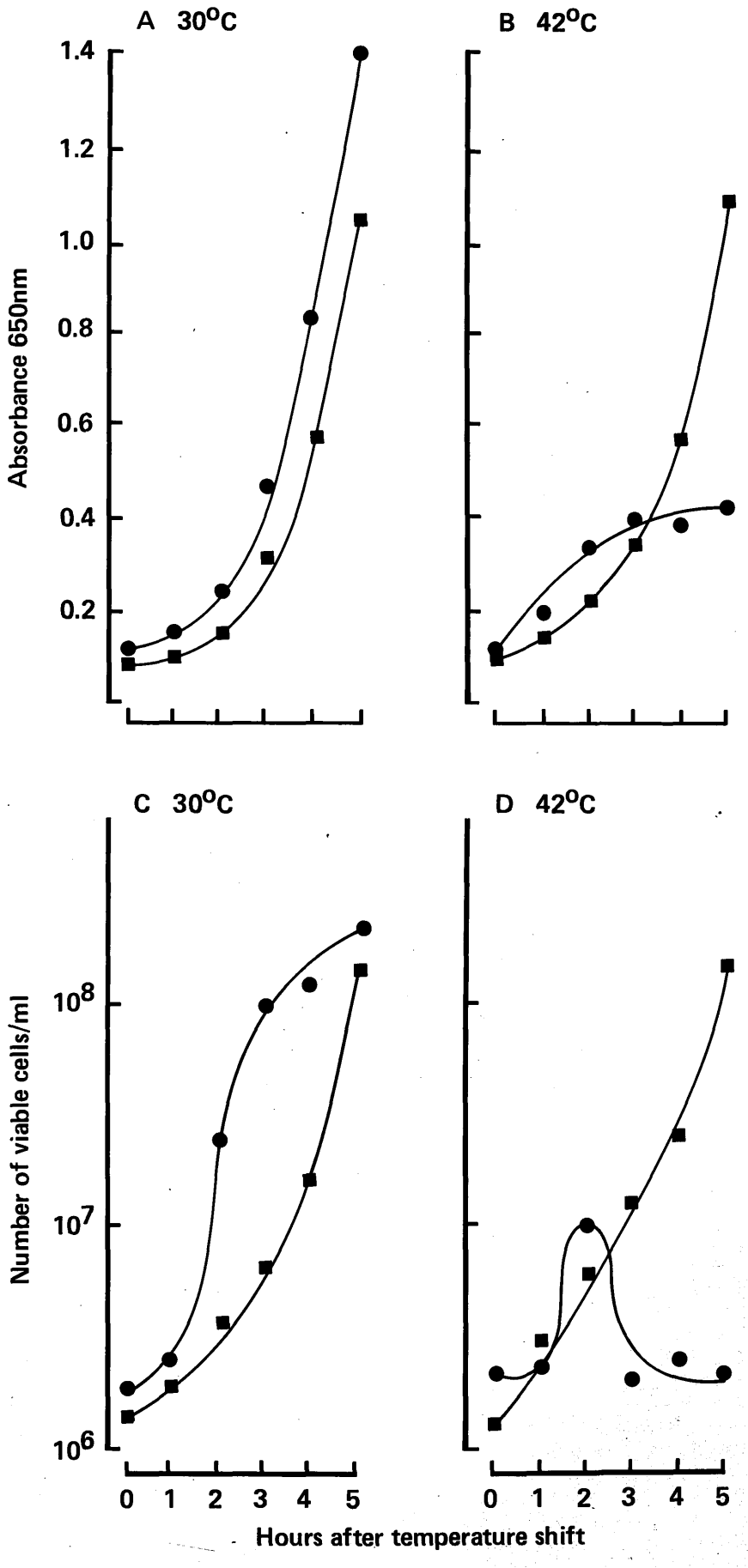

A 30°C
B 42°C
C 30°C
D 42°C
Absorbance 650nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Number of viable cells/ml
10^8
10^7
10^6
0 1 2 3 4 5
0 1 2 3 4 5
Hours after temperature shift

Figure 8: Verification of the phenotypes of strains HD152 trp+ and KH54 trp+.

Media composition	Growth of revertants	
	HD152	KH54
M9 complete	yes	yes
M9 -trp	yes	yes
M9 -(trp and thr)	no	no
M9 -(trp and his)	no	no
M9 -(trp and thy)	no	no
M9 -(trp and ilv)	yes	no
M9 -(trp and met)	yes	no
M9 -(trp and tyr)	no	no

The strains HD152 trp+ and KH54 trp+ were grown on M9 plates containing thr, trp, his, ilv, met and/or tyr as determined above. Triplicate plates were incubated at 30°C overnight.

this construct. Therefore, only the data for plasmids pIF117 and pIF211 are shown. Figure 35 shows that the rho phenotype of HD117 and HD211 was unaltered. It is fortuitous that successful analysis of HD211 occurred since computer analysis data is avaliable for plasmid pIF211 DNA sequence.

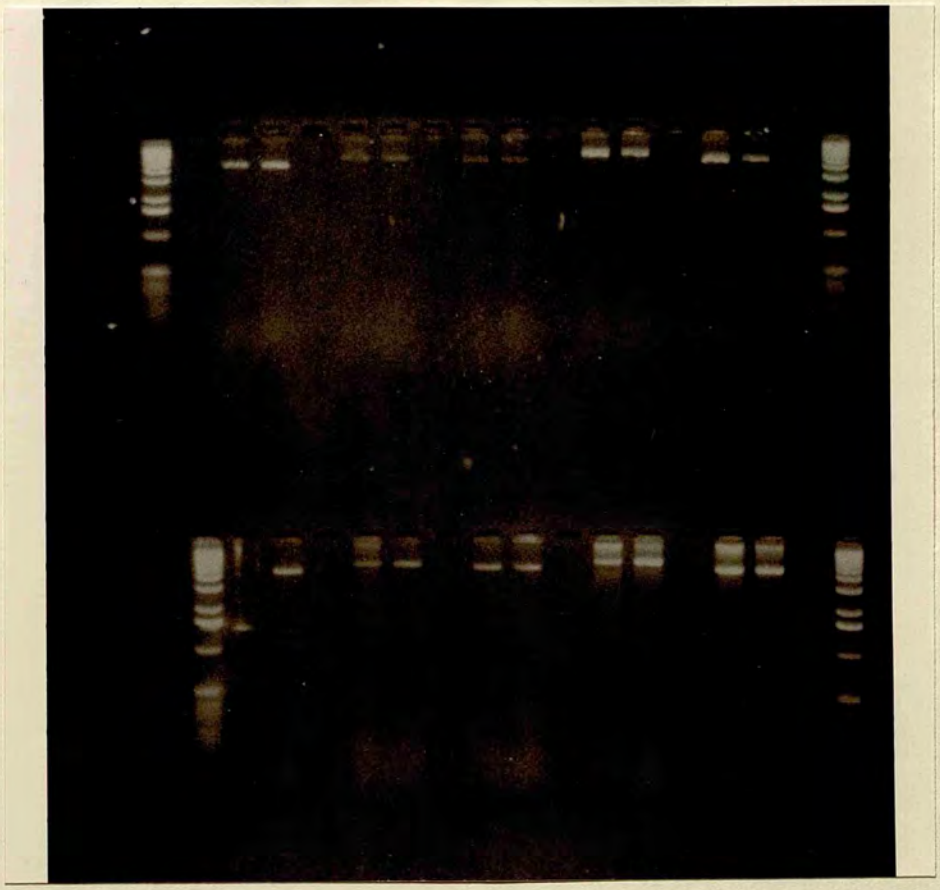
3. Analysis of the interferon mRNA:

Cultures KH211, KH117, HD211 and HD117 were grown for three-four hours at 30°C in minimal medium to ensure that they had entered exponential growth phase. They were then left to grow for a further 2 hours at 42°C before total RNA was isolated. Samples were glyoxalated, Northern blotted then hybridised to α1 (3×10^7 dpm/μg) or α2 (4×10^7 dpm/μg) specific probes. The results are shown in Figure 36. All mRNAs produced by KH117 are also produced by HD117. Therefore, it would appear that all the α1 interferon mRNAs synthesised in this construct are terminated at rho-independent sites. It would be interesting to determine, by computer analysis of the plasmid DNA sequence, if these are characterised by stem-loop structures. In contrast, one mRNA species produced by KH211, the smallest, is not present in mRNA profiles of HD211. This mRNA species appears to terminate in vivo at a rho-dependent site. The other three mRNA species produced by KH211 and HD211 are terminated at rho-independent sites. Computer analysis of the DNA sequence data of construct pIF211 predicted the formation of five stem loop structures which are all well downstream from the region shown in Figure 26 to give rise to the termination of this mRNA species. Rho-independent termination observed at the other three positions correlates with computer predicted stem loop structure formation in the DNA sequence.

4. Attempts to determine the 3' end of the α2 mRNA terminated at the rho-dependent termination site:

Rho-dependent terminators are ill defined. The most widely accepted model has rho bind to a region of RNA lacking in secondary structure that is 60-100 base pairs long. (Figure 2.) NTP hydrolysis results in the release of the

Figure 34: Identification of plasmid DNA inserts in strains HD152 trp+ and KH54 trp+.



Plasmid DNA prepared as described in General Methods 3. was linearised by digestion with PstI. The samples were then electrophoresed on a TBE 1% agarose gel. Samples were run in duplicate.

Plasmids transformed into strain KH54 trp+ are shown in the top half of the gel. From left to right; pIF213, pIF212, pIF211, pTH172 and pIF117. Plasmids transformed into strain HD152 trp+ are shown in the bottom half of the gel. From left to right; pIF213, pIF212, pIF211, pTH172 and pIF117.

The 1 kilobase DNA ladder is shown on the extreme left and right of the top and bottom sections of the gel.

Figure 35: The effect of temperature on the growth of HD211 and HD117 rho temperature sensitive strains in polypeptone glucose medium.

HD211 (o) and HD117 (□) cultures growing exponentially at 30°C were divided in half. One half was kept at 30°C (A and C) while the other half was transferred to 42°C for further incubation (B and D). Cell growth was measured by asborbance at 650nm (A and B) and by colony formation (C and D).

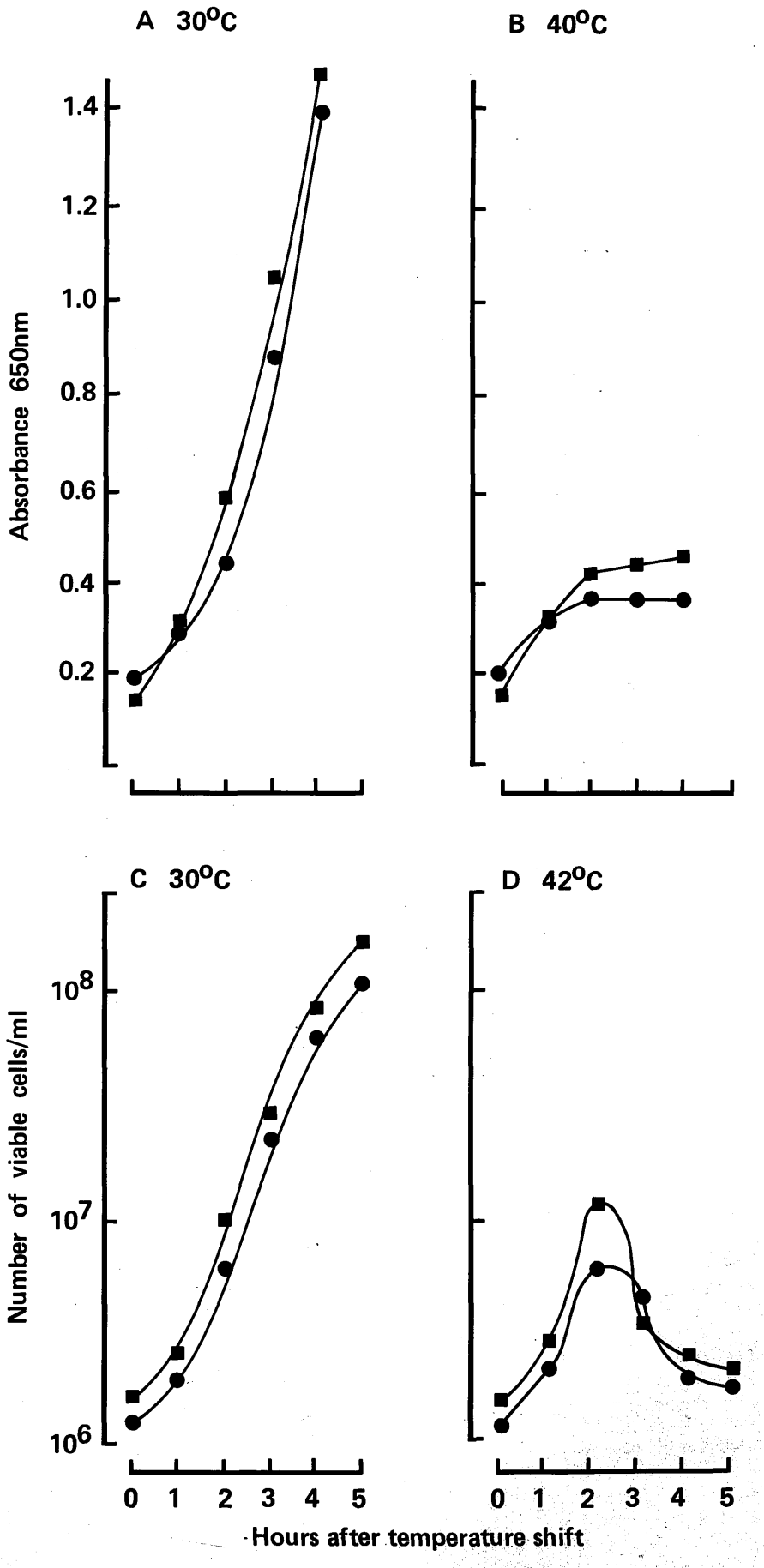
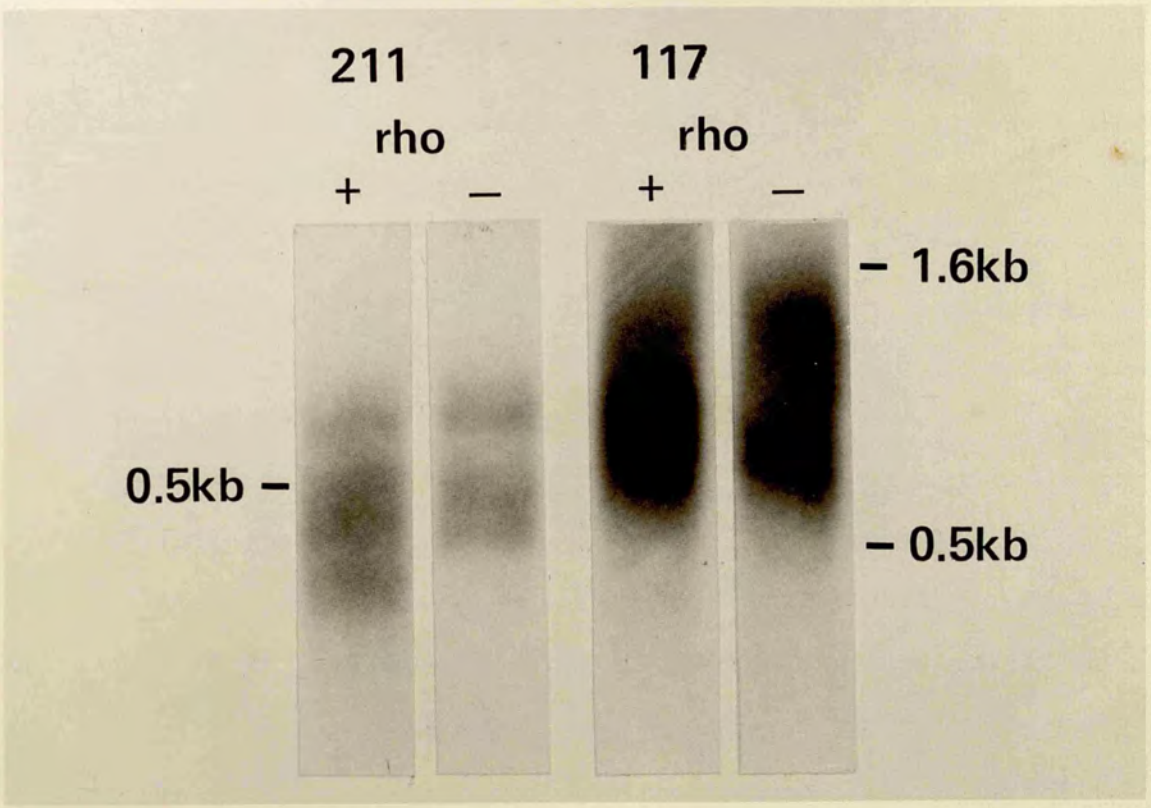

A 30°C
B 40°C
C 30°C
D 42°C
Absorbance 650nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Number of viable cells/ml
10^8
10^7
10^6
0 1 2 3 4 5
0 1 2 3 4 5
Hours after temperature shift

Figure 36: Comparison of interferon mRNA termination in rho+ and rho- strains.



Total cellular RNA (8µg) was isolated from rho+ and rho- strains containing plasmids pIF211 and pIF117 was probed with a nick translated 853 base pair α2 DNA fragment (strains containing pIF211) and a 676 base pair α1 DNA fragment (Strains containing pIF117). The probes were prepared as described in Methods 3, (a), (i) and (b) (i).

Lane 1, KH211 (+). Lane 2, HD211 (-). Lane 3, KH117 (+). Lane 4, HD117 (-).

DNA size markers, 1.6 and 0.5 kilobases, are shown on the extreme right.

transcript from the paused RNA polymerase molecule. (von Hippel et al. 1984.) Rho-dependent termination sites are usually expected to be found in non-translated regions-at the end of operons or in intercistronic regions. Termination often occurs over a range of bases (Wu et al.1981).

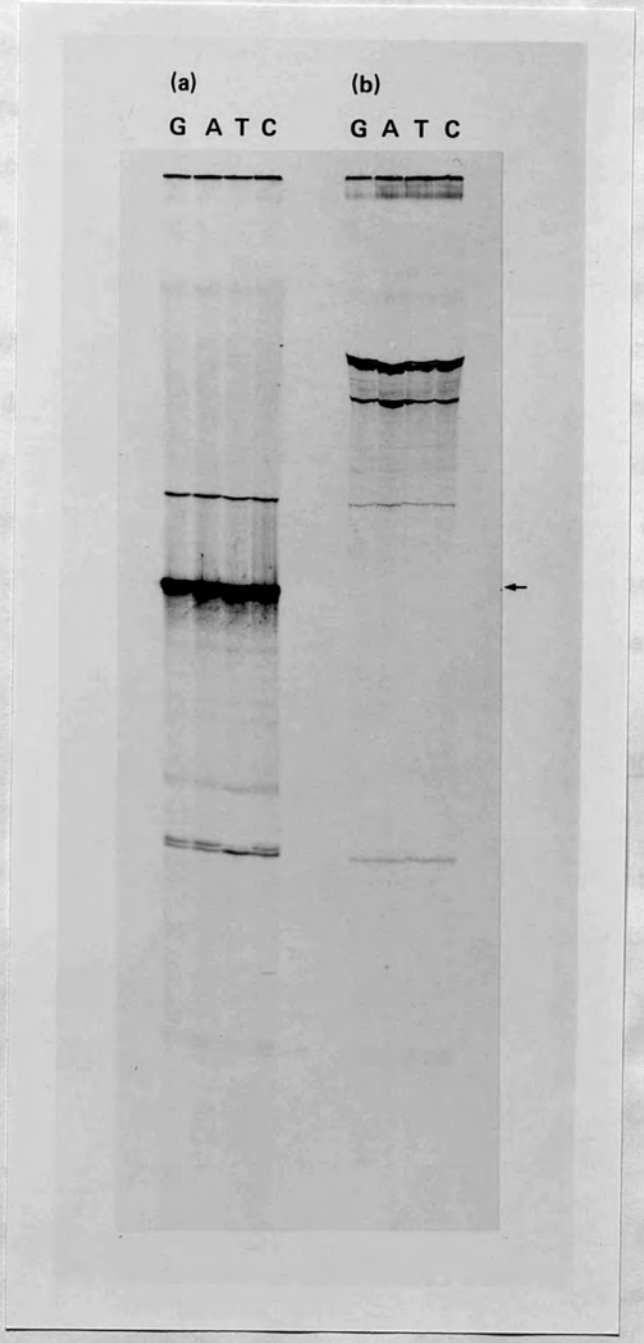
The mRNA species produced by KH211 that has been shown to be terminated at a rho-dependent site contains a translated sequence that is common to all α2 interferon mRNAs. When the sequence is translated it produces the N terminal region of the α2 interferon protein. The fact that this species is contained in all other α2 interferon mRNA makes determination of the 3' end difficult and not possible by conventional methods for RNA sequencing. Two methods using modified Sanger dideoxy sequencing were used. The 193 base pair fragment obtained by BglII/EcoRI digestion of pIF211 (Figure 6) corresponding to the first 193 base pairs of the RNA coding sequence and extending beyond the 3' terminus of the small mRNA, was used as template for incorporating deoxy/dideoxy nucleotides by the Klenow fragment of DNA polymerase I. Since the larger α2 interferon mRNAs are longer than the 193 base pair fragment, the Klenow fragment is incapable of being used for sequencing the RNA. No successful sequence was determined since, (Figure 37 (a)) instead of the typical sequencing patterns, coincident radiolabelled bands of discrete sizes are present in all four tracks. A similar experiment carried out with the adjacent BglII/BglII 260 base pair fragment (Figure 6), which, using the same rationale, should have provided sequence data for the next larger mRNA species, also produced uniform banding patterns (Figure 37 (b)). Therefore, although this method was theoretically applicable for some unknown reason no explainable results were obtained.

An alternative method was attempted involving purification of the smallest α2 interferon mRNA species from all the others. The 260 base pair DNA fragment obtained from BglII digestion of pIF211 (Figure 6) was coupled to epoxy-activated cellulose (Methods 7, (d), (ii)). All α2 mRNAs except the

Figure 37: RNA sequence analysis of the smallest interferon α2 mRNA using specific DNA fragments.

Total cellular RNA (8µg) was annealed to (a) a 193 base pair DNA fragment and (b) a 260 base pair DNA fragment prepared as described in Methods 3, (b), (ii). The sequence was extended by the Klenow fragment of DNA polymerasae I by the method of Sanger et al. (1977).

The four tracks in both (a) and (b) are from left to right; G, A, T and C.


(a)
G A T C
(b)
G A T C

smallest species contain this region and should be retained on the column. The smallest α2 interferon mRNA species is not bound to the column and co purifies with the ribosomal RNA. This semi-purified preparation was used as primer in the dideoxy sequencing reaction. In this case the 193 base pair fragment generated by BglII/EcoRI digestion of pIF211 (Figure 6) was incorporated into M13. The autoradiograph is shown in Figure 38. As before, no interpretable information was obtained as each track appears identical until the point indicated by the arrow where a sequence is generated. Unfortunately, the smeared background makes the sequence unreadable and is thought to be due to the presence of the more abundant contaminating ribosomal RNA. There is no obvious explanation for the coincident banding pattern. Therefore attempts to determine the precise 3' end of the message were unsuccessful. However, as stated earlier in Results and Discussion, B, 4 this species is not detected by the interferon α2 260 base pair fragment (Figure 6) and so it must be less than 218 base pairs in length.

E. Effects of altering arginine coding sequences on rho-dependent termination in α2 interferon mRNA:

The genetic code shows redundancy with several different nucleotide triplets encoding the same amino acid. Workers analysing published DNA sequence data have reported a strong correlation bewteen codon usage in highly expressed genes and relative tRNA abundance in the host. (Grosjean and Fiers 1982.) Both α1 and α2 interferon contain a significant percentage of codons rarely used by E.coli. This is particularly true for arginine residues. In α2 all the arginines are encoded by rare tRNAs. Within the first twenty amino acids of the α2 sequence these rare arginine tRNAs are required four times in two blocks of two. In the α1 sequence the rare arginine tRNAs are required three times only, the second in the last block of two present in the α2 sequence is absent. (Figure 39.) It is possible that, because of the rarity of

Figure 38: RNA sequence analysis of the smallest mRNA produced by interferon α2 using M13 templates containing the 193 base pair DNA fragment.

Semipurified RNA (Methods 7, (d), (ii)) was annealed to the M13 template and extended using the Klenow fragment of DNA polymerase I by the method of Sanger et al. (1977).

The four lanes contain, from left to right, G, A, T and C. The arrow indicates the position where the sequence ladder begins.

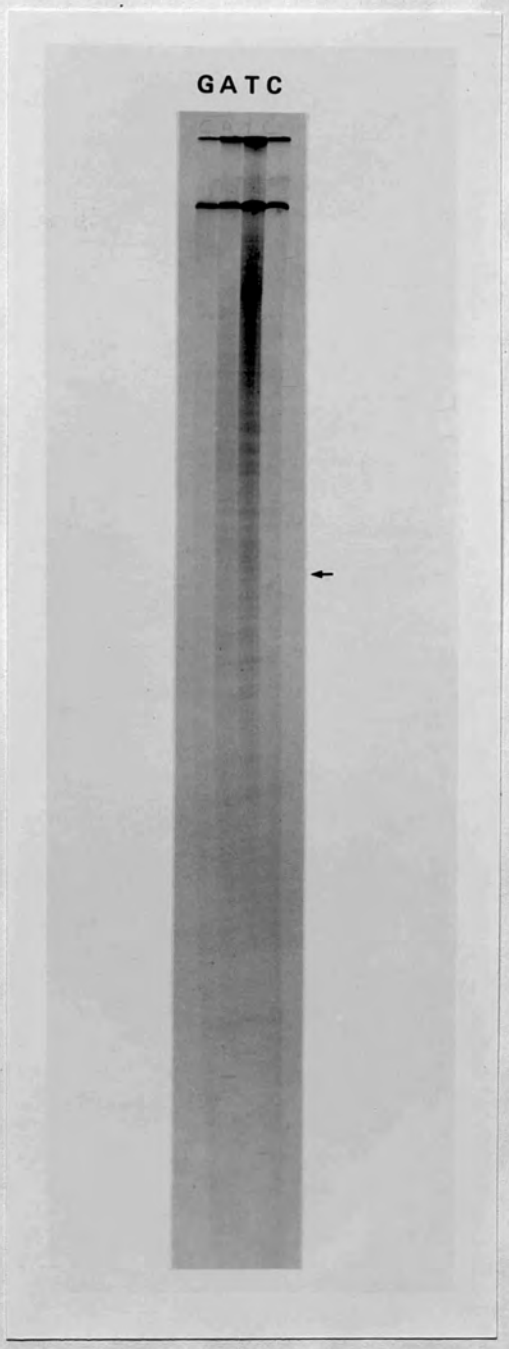

GATC

Figure 39: Model for rho-dependent termination within the α2 interferon coding secence.

Interferon α2 and α1 sequences showing the possible ribosome pause sites and the potential secondary structure formation within the region of DNA sequence shown to produce rho-dependent termination in interferon α2.

Interferon $\alpha 2$ sequence:

A T G T G C G A T C T G C C T C A A A C C C A C A G C C T G G G T A G C A G G

A G G A C C T T G A T G C T C C T G G C A C A G A T G A G G A G A A T C T C T

C T T T T C T C C T G C T T G A A G G A C A G A C A T G A C T T T G G A T T T

C C C C A G G A G G A G T T T G G C A A C C A G T T C C A A A A G G C T G A A

A C C A T C C C T G T C C T C C A T G A G A T G A T C C A G C A

Interferon $\alpha 1$ sequence:

A T G T G T G A T C T C C C T G A G A C C C A C A G C C T G G A T A A C A G G

A G G A C C T T G A T G C T C C T G G C A C A A A T G A G A A T C T C T C C T

A
G . T
T . G
C – G
T – A
G – C
T – A
C – G

T C C T C A C A T G A C T T T G G A T T T C C C C A G G A G G A G T T T G A

T G G C A A C C A G T T C C A G A A G G C T C C A G C C A T C T C T G T C C T

C C A T G A G C T G A T C C A C C A

33054-7

the appropriate tRNAs, a ribosome translating the interferon message (α1 and α2) travels more slowly through these regions. This slow movement of the ribosome may enable the polymerase to transcribe well ahead, decoupling transcription and translation and providing, in the case of α2 interferon producers, a long region of message free from secondary structure and ribosomes. If the polymerase pauses at any distant site it creates an environment consistent with that propos ed to give rise to rho binding and rho-dependent termination. Such a rho dependent site has been identified (Results and Discussion B, 4 and D, 3). Rho binding is not possible in the same region of the α1 interferon message because of the formation of a stem-loop structure. (Figure 27.) Bonekamp et al. (1985) have postulated such a mechanism to explain the production of different mRNA species from an artificial leader DNA sequence preceeding the pyrE attenuator.

The four arginine codons recognised by rare E.coli transfer RNAs in α2 mRNAs were considered as possible sites for ribosome pausing and subsequent uncoupling of transcription and translation. In order to test this hypothesis two thirty base oligomers were synthesised such that the AGG and AGA arginine codons were replaced with CGTs, the most commonly used E.coli arginine codons (Methods 6). These oligomers were then introduced into the plasmid to replace the authentic regions by site-directed mutagenesis techniques. (Figure 9.)

Unfortunately no ampicillin resistant, tetracycline resistant colonies were obtained when both oligomers were present in the reaction mix. However, to test that the method was operable Fragment 1 was self-ligated and shown to produce ampicillin sensitive and tetracycline resistant colonies. Self-ligated Fragment II produced ampicillin resistant, tetracycline sensitive colonies. This indicated that ligation and transformation was indeed occurring. When the oligomers were added separately to reactions

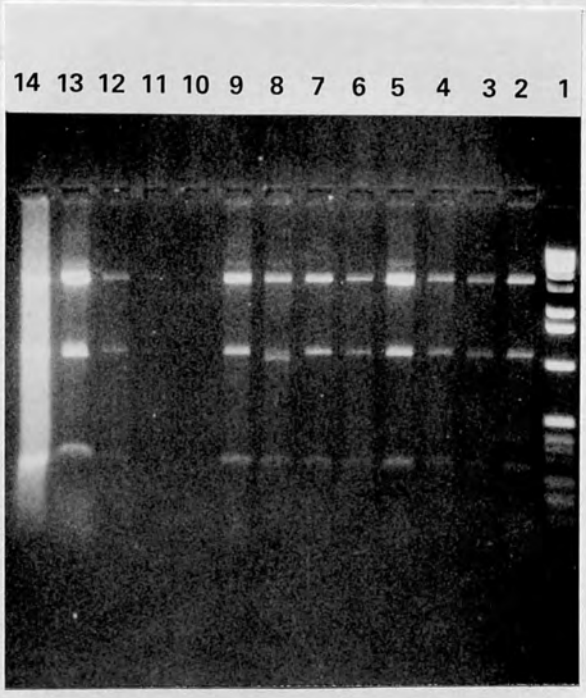
transformants were produced with oligomer 1 but not oligomer 2. It is possible that some residual contaminant in oligomer 2 inhibited the action of Klenow in the reaction mix.

Only 11 ampicillin and tetracycline resistant transformants were obtained with oligomer 1. The poor yield of ampicillin and tetracycline resistant transformants may result from difficulties experienced by the Klenow fragment of DNA polymerase I in making the sequence double stranded. Reverse transcriptase was used in place of the Klenow fragment of DNA polymerase 1 but it failed to produce any suitably resistant transformants.

Introduction of oligomer 1 into the interferon sequence was expected to create an additional RsaI site. RsaI digestion of the unaltered pIF213 produced three fragments, 245, 1094 and 2977 base pairs long. However, RsaI digestion of the plasmid containing oligomer 1 should produce four fragments, 76, 245, 1016 and 2977 base pairs in length. Plasmid DNA was isolated from the 11 transformants obtained and a sample digested with RsaI. The RsaI restriction maps are shown in Figure 40. Although it is not possible to easily detect the 76 base pair fragment generated by the additional RsaI site on agarose gels, it is possible to see the difference in size between the 1094 base pair fragment in the unaltered plasmids and the 1016 base pair fragment in the oligomer 1 containing plasmid. All of the transformants, except one, had a restriction map expected for wild-type pIF213. However, transformant 7, has the 1016 base pair fragment rather than the 1094 base pair fragment and therefore contains the oligomer 1.

Total RNA was extracted from cultures of transformants 1, 7 and 11. (1 and 11 are unchanged while 7 contains two CGT codons in place of two AGG codons.) The RNA was glyoxalated, Northern blotted and hybridised to the 853 base pair $\alpha 2$ interferon probe (1.5×10^7 dpm/μg). Figure 41 shows that the smallest mRNA is indeed not present in 7, the plasmid containing the

Figure 40: Analysis of RsaI digestion profiles of plasmid DNA isolated from transformants obtained as a result of site-directed mutagenesis of construct pIF213.

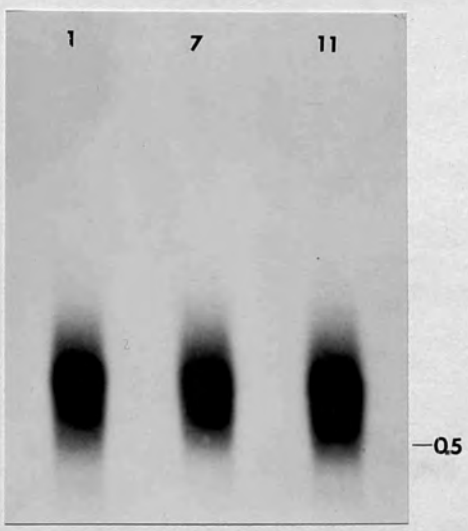


Plasmid DNA (0.5µg) was digested with RsaI as described in Methods 6, (d). The samples were electrophoresed on a TBE 1% agarose gel containing ethidium bromide and visualised under U.V 300nm.

Lane 1, 1 kilobase DNA ladder. Lane 2, transformant 1. Lane 3, transformant 2. Lane 4, transformant 3. Lane 5, transformant 4. Lane 6, transformant 5. Lane 7, transformant 6. Lane 8, transformant 7. Lane 9, transformant 8. Lane 10, transformant 9. Lane 11, transformant 10. Lane 12, transformant 11. Lane 13, transformant 7 (2ug). Lane 14, unaltered pIF213 (1ug).

mutated region but is present in 1 and 11. It appears that replacement of two of the rare arginine codons from this region with the more commonly used E.coli codons does reduce transcription termination at the rho-dependent site, as hypothesised, by reducing ribosome pausing due to the rare codon content of the DNA sequence.

Figure 41: Comparison of mRNA produced by unaltered construct pIF213 and construct pIF213 containing fewer rare arginine codons.



Total cellular RNA (8µg) isolated from cultures containing pIF213 transformant 1, pIF213 transformant 7 and pIF213 transformant 11 were probed with a nick translated 853 base pair DNA fragment prepared as described in Methods 3, (b), (i).

Lane 1. pIF213 transformant 1. Lane 2. pIF213 transformant 7. Lane 3 pIF213 transformant 11.

A DNA size marker, 0.5 kilobases, is shown on the extreme right.

IV: GENERAL CONCLUSIONS

IV. General Conclusions:

The results obtained from this investigation show that DNA sequences can influence markedly the production and stability of mRNA.

Production of α1 interferon varied appreciably in the four constructions examined. While pGW17 and pIF117 were expected to produce differing amounts of protein, increased interferon production was not anticipated when the threonine attenuator operon was added to the 3' end of the interferon sequence.

Plasmid copy number studies showed that the introduction of the α1 interferon sequence into plasmids pBR322 and pAT153 increased copy number. Constructions containing the α1 sequence had twice as many plasmid copies per cell as those without the α1 sequence.

The synthesis of multiple α1 interferon mRNAs was not expected. The species resulted from the utilisation of one promoter and several termination sites. The sequence while containing all the information required to code for the mature protein, contained very little non coding sequence and although termination occurred at several places within this region, it was not 100% efficient. Several long readthrough mRNA species were produced. These species terminated in the vector sequence at the β-lactamase terminators and yielded polycistronic messages coding for interferon and β-lactamase. The half-lives of all the messages produced by pGW17 and pIF117 were identical (1-1.5 minutes).

Introduction of the threonine attenuator operon was possible in two opposing orientations. The resulting constructions pTH171 and pTH172 produced only one major mRNA species when transcription products were isolated from strains grown in the presence of threonine. The threonine attenuator was capable of effecting termination when inserted in either orientation. Termination appeared to be more efficient when the termination sequence was inserted in the non-functional orientation (pTH171). Only one

mRNA species was produced by this construct while several readthrough species could be detected in RNA isolated from the construction containing the attenuator inserted in the functional orientation (pTH172). The mechanism by which the attenuator precludes termination at sites in the non-coding sequence known to be effective in pGW17 is not understood.

The half-lives of the major mRNA species produced by pTH171 and pTH172 were significantly different. The species produced by pTH171 had a half-life of 4 minutes compared with the major pTH172 mRNA which had a half-life greater than 8 minutes. This very stable species produced by pTH172 displayed anomolous behaviour on denaturing gels. Differential probing produced a complex set of results. Several of the internal interferon probes failed to hybridise to the mRNA. Either the mRNA did not contain these sequences or it formed a resistant secondary structure. Regions involved in the formation of the secondary structure would necessarily be involved in base pairing and unavaliable for interaction with the single stranded DNA probes. These regions would therefore, be undetected. Attempts to determine the 5' end of the message failed to define the exact transcription initiation site but confirmed that the complete coding sequence was contained within the seemingly small mRNA.

Plasmids containing the $\alpha 2$ sequence, differing in selected regions of the non-coding sequence were developed in an attempt to obtain commercially significant quantities of $\alpha 2$ interferon. At best, $\alpha 2$ production achieved similar levels per plasmid copy as $\alpha 1$ production but, in absolute terms, it was considerably less since $\alpha 2$ plasmids had lower copy numbers.

Plasmid copy numbers of all the $\alpha 2$ constructs were similar to the vector in which they were based. While altering selected regions of the non coding sequence affected protein production, copy number was not affected.

A significant difference between the plasmids containing the two interferon sequences was their stabilities. All the $\alpha 1$ containing plasmids

were 100% stable over 40 generations. Plasmid pIF217 was 100% stably maintained but all of the other α2 containing plasmids were unstable to a greater or lesser extent. The degree of instability increased with increased interferon production.

Multiple mRNA species were also produced by the α2 constructions. A comparison of the quantities of mRNA produced revealed that two, pIF210 and pIF212, produced significantly less than the others. These two constructions contain a deletion 5' to -35 region of the trp promoter and it appears that this region is important for promoter function.

In constructions containing the complete α2 non-coding sequence termination occurred efficiently within this region and no mRNA containing vector sequence were detected. In those α2 constructions containing smaller amounts of non-coding sequence (similar to the al constructs pGW17 and pIF117) long readthrough mRNA species were detected. Production of the long mRNAs in both the α1 and α2 constructions was accompanied by a concomitant accumulation of β-lactamase. Suprisingly, the α1 and α2 polycistronic messages terminated preferentially at different pBR322/pAT153 termination sites.

Among the many mRNA species produced by the α2 interferon constructions there were several that appeared too small for translation to yield mature α2 interferon. Unlike the apparently small mRNA species produced by construct pTH172, this was not due to secondary structure considerations. Indeed, three small mRNA species were shown to terminate prematurely within the coding sequence. In contrast to α2, transcription of the α1 sequence produced no prematurely terminated mRNA species. Computer analysis of the α2 sequence revealed two positions where stem loop structures might form. Such structures are associated with rho-independent termination and RNA polymerase pausing. The areas predicted to form these stem loops coincided with the region shown to contain two termination sites

by analysis of mRNA profiles of HD211 and KH211 rho+ and rho- strains. The computer did not predict a termination site for the smallest mRNA species. Comparisons of mRNA profiles obtained from rho+ and rho- strains transformed with pIF211 revealed that the smallest mRNA species was terminated at a rho-dependent termination site.

It was proposed that the prematurely terminating mRNA species produced by the α2 constructions were due to transcription/translation decoupling by ribosome stalling at rare arginine codons. This enabled rho to bind to the unstructured RNA downstream from the stalled ribosome. No such rho-dependent termination was obtained for the α1 constructions because the RNA was able to form competing secondary structures in the region required for rho binding. The rare arginine codons in the early coding sequence were replaced with commonly used arginine codons by site-directed mutagenesis. Termination at the rho-dependent site was severely limited in the mutated construction.

Half-life studies showed that all species except the readthrough mRNA produced by pIF217 decayed uniformly with half-lives of 1-1.5 minutes. The readthrough species produced by pIF217 appeared resistant to degradation for the first 2 minutes, then they decayed rapidly, being hardly detectable 4 minutes after rifampicin addition No delayed onset of decay was observed for readthrough species produced by pIF212, pIF213 or any the α1 constructions.

The affects of growth-rate and growth phase on mRNA stabilty and species production were determined for pIF217. When the half-lives of the interferon mRNA produced in M9CA and MOPs glucose were compared they showed a similar pattern of decay. These media produced similar growth rates. All mRNA except the readthrough species decayed uniformly with half lives of 1-1.5 minutes. The readthrough species apparently increased in abundance until 2 minutes after rifampicin addition when they decayed

uniformly with half-lives of 2 minutes. Polypeptone glucose medium which supports faster growth, did not elicit the same mRNA behaviour. The readthrough mRNA did not increase in abundance but decayed uniformly as did the other interferon mRNA species. Interferon production was highest in cultures grown in the polypeptone medium. No obvious relationship exists between mRNA stability and growth rate.

When the half-lives of the interferon mRNA were determined at varying growth phases in MOPs glucose medium both the mono- and polycistronic mRNAs were produced at all three phases but the proportion of one type to another and the relative stabilities of the two types varied. The predominant species produced in lag phase was the readthrough product. At the other two growth phases, production of the monocistronic species was preferred. The readthrough species apparently increased in abundance when isolated from exponential and early stationary phase cultures but decayed uniformly when isolated from cultures in lag phase.

It is apparent from this investigation that although manipulation of selected non-coding sequences such as the ribosome binding site, regions 5' to the -35 region of the promoter and the addition of an effective termination signal immediatley downstream from the inserted sequence can give rise to increased protein production, expression of foreign proteins may be limited in some systems by coding sequence content. Important features to consider when expression of a foreign protein is required in E.coli include the selection of an efficient promoter and a suitable ribosome binding site. Vectors chosen must be capable of maintaining the insert stab ly. Sequences should contain as few rarely used E.coli codons as possible and an efficient termination signal should be inserted immediately downstream of the introduced sequence.

APPENDICES

Appendix A:

1. E.coli strain MM294.

Genotype:endoI-, thi-1-, hsr^k-, hsm^k+

2. E.coli strain DH1.

Genotype: F-, recA1, endA1, gyrA96, thi-1, hsdR17(r_k^- m_k^+) supE44, recA1?, λ-

References: Low (1968). Meselson and Yuan (1968).

3. E.coli strain JM103.

Genotype: Δ (lac pro), thi,strA, supE, endA, sbcB, $hsdR^-$, F'traD36, proAB, $lacI^q$,Z Δ M15

Reference: Messing et al. (1981).

4. E.coli strain HD152.

Genotype: F-, thr-33. trpE9829, his-213, tyrA15, thyA707, rho-702, λ-,supD126,IN(rrnD-rrnE)1.

Comments: trpE9829 and tyrA15 are amber mutations. rho-702 and supD126 are temperature-sensitive mutations.

References: Imai and Shiqesada (1978) Inoko et al. (1977).

5. E.coli KH54.

Genotype: F-, thr-33. trpE9829, his-213. tyrA15, thyA707. ilv-683. metE46. λ-, supD126, IN(rrnD-rrnE)1.

Comments: supD126 is a temperature-sensitive mutation.

Referrence: Horiuchi and Nagata (1973).

6. E.coli strains HD:

E.coli strain HD152 trp+ containing α1 plasmids (pIF117 and pTH172) and α2 plasmids (pIF210, pIF211, pIF212, pIF213 and pIF217).

7. E.coli strains KH:

E.coli strain KH54 trp+ containing α1 plasmids (pIF117 and pTH172) and α2 plasmids (pIF210, pIF211, pIF212, pIF213 and pIF217).

Appendix B:

A. Liquid media:

1. L-broth (Luria-Bertan Medium):

Per liter:

Bacto-tryptone, 10g

Bacto-yeast extract, 5g

NaCl, 10g

The pH was adjusted to 7.2 with 5M NaOH.

Referrence: Maniatis et al.(1982).

2. M9CA Medium:

Per liter:

Na_2HPO_4, 6g

KH_2PO_4, 3g

NaCl, 0.5g

NH_4Cl, 1g

The pH was adjusted to 7.4, autoclaved and cooled before the addition of sterile:

1M $MgSO_4$, 1ml

25% glucose, 20ml

15% bacto-casamino acids, 20 ml

1% thiamine, 1ml

Referrence: Maniatis et al. (1982).

3. 2 x TY medium:

Per liter:

bacto-tryptone, 16g

bacto-yeast extract, 10g

NaCl, 5g

The pH was adjusted to 7.2 with 5M NaOH.

Referrence: Maniatis et al. (1982).

4. Polypeptone glucose Medium:

Per liter:

polypeptone (peptone 180, Gibco Europe), 20g

NaCl, 5g

Glucose, 5g

The pH was adjusted to 7.2 with 5M NaOH.

Referrence: Inoko and Imai (1976).

5. MOPs-glucose Medium:

A 10x concentrate was prepared by mixing the following solutions in the given order to prevent precipitation of the various salts:

Per 100ml:

1.0M potassium morpholinopropane sulphonate, adjusted to pH7.4 with KOH, 40ml

1.0M N'-Tris (hydroxymethyl) methyl glycine (Tricine), freshly prepared, adjusted to pH7.4 with KOH, 4ml

0.01M $FeSO_4$, freshly prepared, 1ml

1.0M NH_4Cl, 8ml

0.276M K_2SO_4, 1ml

6.0×10^{-4}M $CaCl_2$. 1ml

0.528M $MgCl_2$. 1ml

5.0M NaCl, 10ml

micronutrients (a solution containing 3×10^{-6}M $(NH_4)_6(MO_7)_{24}$; 4×10^{-4}M H^3BO^3; 3×10^{-5}M CoCl; 1×10^{-2}M $CuSO_4$; 8×10^{-5}M $MnCl_2$; 1×10^{-5}M $ZnSO_4$), 1ml

distilled water, 36ml

The solution was sterile filtered.

MOPS-glucose per liter:

10 x MOPs concentrate, 100ml

Sterile 0.132M K_2HPO_4, 10ml

Sterile 20% glucose, 20ml

Sterile distilled water, 870ml

Referrence: Neidhardt et al. (1974).

B. Plates:

Bacto-agar (15g/l) was added to the appropriate medium.

C. Buffers:

1. TAE:

0.04m Tris-acetate (4.84g Tris base, 1.142ml glacial acetic acid)

0.002M EDTA (2ml, 0.5M EDTA, pH8.0)

2. TBE:

0.089M Tris-borate (10.8g Tris base)

0.089M Boric acid (5.5g boric acid)

0.002M EDTA (4ml, 0.5M EDTA, pH8.0)

3. TE:

10mM Tris.HCl pH8.0

1mM EDTA

4. EE;

5mM Tris

2.5mM acetic acid

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