

New genetic sequences, the interferon peptides
of Type 1 encoded by them and the microorganisms
producing these interferons

The present invention relates to new Type I interferons as well as recombinant DNA methods of producing these peptides and products required in these methods, for example, genetic sequences, recombinant DNA molecules, expression vehicles and organisms.

Interferon is a word coined to describe a variety of proteins endogenous to human cells characterised by partly overlapping and partly diverging biological activities. These proteins modify the body's immune response and are believed to contribute substantial protection against viruses. For example, interferons have been classified into three broad species, α -, β -, and γ -Interferon.

Only one subspecies of β - and γ -interferon is known in humans (see, for example, S. Ohno et al., Proc. Natl. Acad. Sci. 78, 5305-5309 (1981); Gray et al., Nature 295, 503-508 (1982); Taya, et al., EMBO Journal 1/8, 953-958 (1982)). On the other hand, several subtypes of α -interferon have been described (See, for example, Phil. Trans. R. Soc. Lond. 299, 7-28 (1982)). The mature α -interferons reveal a maximum divergence of 23% among each other and are about 166 amino acids long. Of note is also a report of an α -interferon having an unusually high molecular weight (26,000

by SDS polyacrylamide gel electrophoresis, described by Goren, P. et al., Virology 130, 273-280 (1983)). This interferon is called IFN- α 26K. It has been found to have the highest known specific anti-viral and anti-cellular activities.

The interferons known to date appear to be effective against various diseases but demonstrate little or no efficacy in many others (see, for example, Powledge, Bio/Technology, March 1984, 215-228, "Interferon On Trial"). Interferons have also been plagued by side effects. For example, in trials of the anti-cancer properties of recombinant α -interferon trials, doses of around 50 million units, which had been believed to be safe on the basis of Phase I trials have been associated with acute confusional states, disabling arthragia, profound fatigue and anorexia, disorientation, seizures and hepatic toxicity. In 1982, the French government stopped trials with α -interferon after cancer patients receiving it suffered fatal heart attacks. At least two cardiac deaths have also been reported in recent American trials. It has become increasingly clear that at least some of the side effects, like fever and malaise, appear to be inherent in the interferon molecule itself and not in impurities present therein.

Due to the great hopes elicited by the interferons, and spurred by the wish to discover yet new interferon-like molecules with decreased side effects, the present inventors set out to search for and produce such new substances.

The present invention therefore relates to certain new interferons of Type I which may contain a leader peptide, and their N-glycosylated derivatives, (called herein omega-interferon, or IFN-omega) which contain 168 to 174, preferably 172, amino acids, and which reveal a divergence of 30 to 50%, preferably

40-48%, compared to the hitherto known subtypes of α -interferon, and a divergence of about 70% compared to β -interferon, while showing similar effectiveness to α -interferons, and which do not reveal many of the known therapeutic disadvantages of these molecules.

The invention therefore provides new interferons, in fully purified form, the unglycosylated and glycosylated forms thereof, genetic sequences coding therefor, as well as recombinant molecules containing the sequences. The invention also provides expression vehicles such as plasmids containing genetic sequences as indicated, as well as various hosts, such as microorganisms or tissue culture hosts, capable of producing the novel interferons by fermentation or tissue culture methods.

In a preferred embodiment, the invention provides omega-interferons as well as the corresponding genetic sequences, having the following formulae:

				5					10					15
Cys	Asp	Leu	Pro	Gln	Asn	His	Gly	Leu	Leu	Ser	Arg	Asn	Thr	Leu
TGT	GAT	CTG	CCT	CAG	AAC	CAT	GGC	CTA	CTT	AGC	AGG	AAC	ACC	TTG
				20					25					30
Val	Leu	Leu	His	Gln	Met	Arg	Arg	Ile	Ser	Pro	Phe	Leu	Cys	Leu
GTG	CTT	CTG	CAC	CAA	ATG	AGG	AGA	ATC	TCC	CCT	TTC	TTG	TGT	CTC
				35					40					45
Lys	Asp	Arg	Arg	Asp	Phe	Arg	Phe	Pro	Gln	Glu	Met	Val	Lys	Gly
AAG	GAC	AGA	AGA	GAC	TTC	AGG	TTC	CCC	CAG	GAG	ATG	GTA	AAA	GGG
				50					55					60
Ser	Gln	Leu	Gln	Lys	Ala	His	Val	Met	Ser	Val	Leu	His	Glu	Met
AGC	CAG	TTG	CAG	AAG	GCC	CAT	GTC	ATG	TCT	GTC	CTC	CAT	GAG	ATG
				65					70					75
Leu	Gln	Gln	Ile	Phe	Ser	Leu	Phe	His	Thr	Glu	Arg	Ser	Ser	Ala
CTG	CAG	CAG	ATC	TTC	AGC	CTC	TTC	CAC	ACA	GAG	CGC	TCC	TCT	GCT
				80					85					90
Ala	Trp	Asn	Met	Thr	Leu	Leu	Asp	Gln	Leu	His	Thr	Gly	Leu	His
GCC	TGG	AAC	ATG	ACC	CTC	CTA	GAC	CAA	CTC	CAC	ACT	GGA	CTT	CAT
				95					100					105
Gln	Gln	Leu	Gln	His	Leu	Glu	Thr	Cys	Leu	Leu	Gln	Val	Val	Gly
CAG	CAA	CTG	CAA	CAC	CTG	GAG	ACC	TGC	TTG	CTG	CAG	GTA	GTG	GGA
				110					115					120
Glu	Gly	Glu	Ser	Ala	Gly	Ala	Ile	Ser	Ser	Pro	Ala	Leu	Thr	Leu
GAA	GGA	GAA	TCT	GCT	GGG	GCA	ATT	AGC	AGC	CCT	GCA	CTG	ACC	TTG
				125					130					135
Arg	Arg	Tyr	Phe	Gln	Gly	Ile	Arg	Val	Tyr	Leu	Lys	Glu	Lys	Lys
AGG	AGG	TAC	TTC	CAG	GGA	ATC	CGT	GTC	TAC	CTG	AAA	GAG	AAG	AAA
				140					145					150
Tyr	Ser	Asp	Cys	Ala	Trp	Glu	Val	Val	Arg	Met	Glu	Ile	Met	Lys
TAC	AGC	GAC	TGT	GCC	TGG	GAA	GTT	GTC	AGA	ATG	GAA	ATC	ATG	AAA
				155					160					165
Ser	Leu	Phe	Leu	Ser	Thr	Asn	Met	Gln	Glu	Arg	Leu	Arg	Ser	Lys
TCC	TTG	TTC	TTA	TCA	ACA	AAC	ATG	CAA	GAA	AGA	CTG	AGA	AGT	AAA
				170										
Asp	Arg	Asp	Leu	Gly	Ser	Ser								
GAT	AGA	GAC	CTG	GGC	TCA	TCT								

In position 111, the sequence GGG (coding for gly) can be replaced with GAG (coding for glu). The preferred molecules also include derivatives thereof which are N-glycosylated at amino acid position 78.

The two omega-interferons mentioned above may, for example, contain the leader peptide of the formula

Met Ala Leu Leu Phe Pro Leu Leu Ala Ala Leu Val
Met Thr Ser Tyr Ser Pro Val Gly Ser Leu Gly.

DESCRIPTION OF THE FIGURES

Figure 1: Restriction map for clone E76E69.

Figure 2: Restriction map for clone P9A2.

Figure 3: DNA sequence of the clone P9A2.

Figure 4: DNA sequence of the clone E76E9.

Figure 5: Genomic Southern Blot Analysis using the DNA of the clone P9A2 as probe

Figure 6: Construction of the expression clone pRHW12

Figure 7: Amino acid and nucleotide differences between Type I interferons

Figure 8a: List of singular nucleotide positions of the IFN- α A gene

Figure 8b: List of singular nucleotide positions of the IFN-omega 1 gene

Figure 8c: List of singular nucleotide positions of the IFN- α D gene

Figure 9: Schematic representation of the synthesis of interferon-subtype of specific samples

Figure 10: Detection of interferon-subtype of specific mRNA's

Figure 11: DNA sequence of the IFN-omega1 gene

Figure 12: DNA sequence of the IFN-pseudo-omega2 gene

Figure 13: DNA sequence of the IFN-pseudo-omega3 gene

Figure 14: DNA sequence of the IFN-pseudo-omega4 gene

Figure 15: corrected list of the IFN-omega gene sequences

Figure 16: Homologies of the signal sequences

Figure 17: Homologies of the "mature" protein sequences

Figure 18: Homologies of the 4 DNA sequences relative to one another

According to the invention, the new omega interferons and the DNA sequences coding for them are obtained as follows:

A human B-cell lymphoid line, for example, Namalwa cells (see G. Klein et al., Int. J. Cancer 10, 44 (1972)) can be stimulated for the simultaneous production of α - and β -interferons through treatment with a virus, for example, Sendai Virus. In the process, the mRNA formed is isolated from the stimulated Namalwa cells, and this can then be used as a template for cDNA synthesis. In order to increase the yield of interferon-specific sequences during the cloning process, the mRNA preparation is separated in a sugar density gradient into regions with different lengths of individual mRNA molecules.

Preferably, the mRNA in the region around 12S (about 800-1,000 bases length of mRNA) is collected. The mRNA which is specific for α -interferons and β -interferons will settle in this region. The mRNA from this gradient

region is concentrated through precipitation and dissolution in water.

The preparation of a cDNA library essentially involves the use of methods known in the literature (see, for example, E. Dworkin-Rastl, M.B. Dworkin and P. Swetly., Journal of Interferon Research 2/4, 575-585 (1982)). The mRNA is primed through the addition of oligo-dT. After that, by adding the four deoxynucleosidetriphosphates (dATP, dGTP, dCTP, dTTP) and the enzyme reverse transcriptase, in an appropriately buffered solution, cDNA is synthesized at 45°C for 1 hour. Through chloroform extraction and chromatography via a gel column, for example, via Sephadex[®] G50, the cDNA/mRNA hybrid is purified. The RNA is hydrolysed by means of alkali treatment (0.3 M NaOH at 50°C for 1 hour), and the cDNA is precipitated with ethanol after neutralising with acid sodium acetate solution. Double strand synthesis is performed after addition of the four deoxynucleosidetriphosphates and E.coli DNA-polymerase I in an appropriately buffered solution, whereby the cDNA is used both as a template and as a primer through hairpin structure formation at its 3' end (6 hours at 15°C) (see also A. Eftratiadis et al., Cell 7, 279 (1976)). Following phenol extraction, Sephadex G50 chromatography and ethanol precipitation, the DNA is subjected, in a suitable solution, to a treatment with endonuclease S1, which is specific to single strands. The hairpin structure as well as any cDNA that was not converted into double stranded is degraded. After chloroform extraction and precipitation with ethanol, the double-stranded DNA (dsDNA) is separated in terms of size on a sugar density gradient. In the subsequent steps of cloning, it is preferred to use only dsDNA of size 600 bp and more in order to increase the probability of obtaining only clones which contain the complete coding sequence for the new interferons.

dsDNA with a length of more than 600 bp is concentrated out of the gradient through precipitation with ethanol and dissolution in water.

In order to increase the number of dsDNA molecules that have been obtained, they are first placed in an appropriate vector, and then introduced into the bacterium E. coli. The vector used is preferably the plasmid pBR322 (F. Bolivar et al., Gene 2, 95 (1977)). This plasmid essentially consists of a replicon and two selection markers. They provide the host with resistance against the antibiotics ampicillin and tetracyclin (Ap^r , Tc^r). The gene for β -lactamase (Ap^r) contains the recognition sequence for the restriction endonuclease PstI. pBR322 can thus be cut with PstI. The overlapping 3'-ends are extended with terminal deoxynucleotide transferase (TdT) along with a premixed quantity of dGTP in an appropriately buffered solution. At the same time, dsDNA is likewise extended with the enzyme TdT, using dCTP at the 3'-ends. The homopolymer ends of the plasmid and dsDNA are complementary and will hybridize if plasmid DNA and dsDNA are mixed in the appropriate concentration ratio and under suitable salt, buffer, and temperature conditions (T. Nelson et al., Methods in Enzymology 68, 41-50 (1980)).

E. coli, HB101 strain (genotype F-, hsdS20 (r - B, m - B) recA13, ara-14, proA2, lacY1, galK2, rpsL20 (S^{mr}), xyl-5, mtl-1, supE44, lambda-) is prepared for transformation of the recombinant vector-dsDNA molecules by means of washing with a $CaCl_2$ solution. Competent E. coli HB101 are mixed with the DNA and, after incubation at 0°C, the plasmid DNA thus obtained is transformed by means of heat shock at 42°C for 2 minutes (M. Dagert et al., Gene 6, 23-28 (1979)). The transformed

bacteria are then spread on tetracyclin-containing agar plates (10 µg per ml). Only E. coli HB101 which have received a vector or recombinant carrier molecule (Tc^r) can grow on this agar. Recombinant vector-dsDNA molecules give the host the genotype $Ap^s Tc^r$ because the introduction of the dsDNA into the β -lactamase gene destroys the information for β -lactamase. Clones are then transferred to agar plates, containing 50 µg/ml ampicillin. Only about 3% grow, meaning that 97% of the clones contain the insertion of a dsDNA molecule. Starting with 0.5 µg dsDNA, more than 30,000 clones were thus obtained; 28,600 clones thereof were individually transferred into the cups of microtiter plates which contained nutrient medium, 10 µg/ml tetracyclin and glycerin. After the clones had grown, the plates were kept at $-70^\circ C$ for storage (cDNA library).

In order to search the cDNA library for the new interferon-gene-containing clones, the clones are transferred after thawing to nitrocellulose filters. These filters rest on tetracyclin-containing nutrient agar. The bacterial colonies are allowed to grow and then the DNA of the bacteria is fixed on the filter.

As a probe, one can advantageously use the insert of the clone pER33 (E. Rastl et al., Gene 21, 237-248 (1983) - see also European Patent application No. 0.115.613) which contains the gene for IFN- $\alpha 2$ -Arg. By means of nick translation, this portion of DNA is marked radioactively, using DNA-polymerase I, dATP, dGTP, dTTP and α - ^{32}P -dCTP. The nitrocellulose filters are first pretreated under relaxed hybridisation conditions, without the addition of the radioactive sample, and after that they are hybridised for about 16 hours with the addition of the radioactive sample. The filters are then likewise washed under relaxed conditions. Due to the low stringency

of hybridisation and washing, not only are clones obtained that contain interferon $\alpha 2$ -Arg, but also other clones containing interferons whose sequences may differ considerably from that of the α -interferon known hitherto. After drying, the filters are exposed on an x-ray film. A blackening effect, which is substantially above the level of background, shows the presence of clones with interferon-specific sequences.

Because the radioactivity signals are of differing quality, the positive clones or the clones that react in a manner leading to a suspicion of positive results are then cultured on a small scale. The plasmid DNA molecules are isolated, digested with the restriction endonuclease PstI, and separated electrophoretically on an agarose gel according to size (Birnboim et al., Nucl. Acid. Res. 7, 1513 (1979)). The DNA in the agarose gel is transferred to a nitrocellulose filter according to the method of Southern (E. M. Southern, J. Mol. Biol. 98, 503-517 (1975)). The DNA in this filter is hybridised with the radio-active, IFN-gene-containing, denatured sample. As positive control is used the plasmid 1F7 (deposited at the DSM under the DSM no. 2362) which contains the gene for interferon $\alpha 2$ - Arg. The autoradiogram clearly showed that two clones, E76E9 and P9A2, contained a sequence that hybridised with the gene of interferon $\alpha 2$ -Arg under nonstringent, relaxed conditions. In order to be able more closely to describe the dsDNA inserts of the clones E76E9 and P9A2, the plasmids of these clones were prepared on a larger scale. The DNA is digested with various restriction endonucleases, for example, with AluI, Sau3A, BglII, HinfI, PstI, and HaeIII. The resulting fragments are separated in an agarose gel. Through comparison with corresponding size markers, for example the fragments which result from digestion of pBR322 with the restriction endonuclease HinfI

or HaeIII, one can determine the sizes of the fragments.

By means of mapping according to Smith and Birnsteil (H. O. Smith et al., Nucl. Acid. Res. 3, 2387-2398 (1967)), one can determine the sequence of these fragments. From the restriction enzyme maps thus determined (Figures 1 and 2), the surprising finding was made that the inserts of the clones E76E9 and P9A2 involve a hitherto unknown interferon gene, that is, the omega-interferon gene.

This information on omega-interferons can be used in order to digest the cDNA insert with suitable restriction endonucleases. The fragments are ligated into the dsDNA form (replicative form) of the bacteriophage M13 mp9 (J. Messing et al., Gene 19, 269-276 (1982)) and are sequenced with the help of Sanger's dideoxy method (F. Sanger et al., Proc. Natl. Acad. Sci. USA 74, 5463-5467 (1977)). The single-strand DNA of recombinant phages is isolated. After the binding of a synthetic oligomer, second-strand syntheses are performed in four separate preparations, using the large fragment of DNA-polymerase I from E. coli (Klenow fragment). For each of the four partial reactions, one of the four dideoxynucleosidetriphosphates (ddATP, ddGTP, ddCTP, ddTTP) are added. This leads to statistically distributed chain breaks at those places where the base that is complementary with respect to the particular dideoxynucleosidetriphosphate happens to be in the template-DNA. Radioactively marked dATP is also used. After termination of the synthesis reactions, the products are denatured and the single-strand DNA fragments are separated according to size in a denaturing polyacrylamide gel (F. Sanger et al., FEBS Letters 87, 107-111

(1978)). The gel is then exposed to x-ray film. From the autoradiogram one can read off the DNA sequence of the recombinant M13 phage. The sequences of the inserts of the various recombinant phages are processed by means of suitable computer programs (R. Staden, Nucl. Acid. Res. 10, 4731-4751 (1982)).

Figures 1 and 2 reveal the strategy of sequencing. Figure 3 shows the DNA sequence of the insert of the clone P9A2; Figure 4 shows that of the clone E76E9. The noncoding DNA strand is shown in the 5'→3' direction, together with the amino acid sequence derived therefrom.

The isolated cDNA of the clone E76E9 for omega(Glu)-interferon is 858 base pairs long and has a 3' nontranslated region. The region which codes for mature omega(Glu)-interferon extends from nucleotide 9 to nucleotide 524. The isolated cDNA of the clone P9A2 for omega(Gly)-interferon is 877 base pairs long, whereby the sequence that codes for mature omega-interferon extends from nucleotide 8 to nucleotide 523. The 3' nontranslated region in the case of P9A2 extends to the poly-A segment.

The DNA sequences that code for mature omega-interferon are completely contained in the clones E76E9 and P9A2. The start at the N-terminal end with the amino acids cysteine-aspartic acid-leucine. Quite surprisingly, the two shown mature omega-interferons are 172 amino acids long; this clearly deviates from the hitherto known length of other known interferons, that is, 166 (or 165) amino acids for α -interferons. Quite surprisingly, the two omega-interferons have a potential N-glycosylation site at amino acid position 78 (asparagine-methionine-threonine).

A comparison of the DNA of the clones E76E9 and P9A2 yields one difference. The triplet in

clone E76E9 which codes for amino acid 111 is GAG and codes for glutamic acid. This triplet in clone P9A2 is GGG, and codes for glycine. The two omega-interferon proteins therefore differ from each other by one amino acid and are referred to herein as omega(Glu)-interferon (76E9) and omega(Gly)-interferon (P9A2).

A comparison of the two omega-interferons with the hitherto known human α -interferon subtypes gives the following picture:

	omega	alpha
Length of protein in amino acids	172	166*
Potential N-glycosylation site at position	78	- **

* Interferon alpha A has only 165 amino acids.

** Interferon alpha H has a potential N-glycosylation site at position 75 (D. Goeddel et al., Nature 290, 20-26 (1981)).

E. coli HB 101 with the plasmid E76E9 and E. coli 101 with the plasmid P9A2 are deposited at the German Collection for Microorganisms (DSM Göttingen) under the numbers DSM 3003 and 3004, respectively, on 3rd July 1984.

To prove that the newly discovered clones produce an activity resembling interferon, the clone E76E9 is cultivated, for example, and the lysate of the bacterium is grown and then tested in a plaque reduction test. As expected, the bacterium produced interferon-like activity (see Example 3).

Moreover, to prove that the two newly discovered interferons are members of a new interferon family, all the DNA was isolated from Namalwa cells and digested with various restriction endonucleases.

In this way it is possible to access the number of genes which are coded by the cDNA's of the clones P9A2 and E76E9. For this purpose, the DNA fragments obtained are separated on agarose gel using the method of Southern (E.M. Southern et al., J. Mol. Biol. 98, 503-517 (1975)), placed on nitrocellulose filters and hybridised under relatively stringent conditions with radioactively labelled specific DNA of the clone P9A2.

The results obtained under hybridisation with DNA's from the plasmid P9A2 and pER33 are shown in Figure 5a:

The individual traces are marked with letters to indicate the various DNA samples digested (E=EcoRI, H=HindIII, B=BamHI, S=SphI, P=PstI, C=ClaI). The left-hand half of the filter was hybridised with the interferon- α probe ("A") and the right-hand half was hybridised with the cDNA insert of the clone P9A2 ("O"). The set of bands which hybridise either with the α -interferon probe or with the new interferon probe, are different. No cross-hybridisation could be detected with the two different probes by assessing the corresponding traces.

Figure 5b illustrates the cDNA of the clone P9A2 and the fragment used for hybridisation. The points of intersection of some restriction enzymes are shown (P=PstI, S=Sau3A, A=AluI). The probe includes only two of the three possible PstI fragments. The hybridisation pattern shows only one hybridising fragment with approximately 1300 base pairs (bp), which belongs to the homologous gene. The shorter fragment, 120 bp long, had run out of the gel. The band belonging to the 5' part

of the gene cannot be discovered since the probe does not contain this region. At least 6 different bands can be discovered in the PstI trace. This means that some other genes which are related to the new sequences must be present in the human genome. If one or more PstI points of intersection are present in these genes, one can expect to be able to isolate at least three more additional genes.

These genes may preferably be isolated by hybridisation from a human gene library contained in a plasmid vector, phage vector or cosmid vector (see Example 4e).

At this point it should be mentioned that the omega-interferons according to the invention do not only encompass the two mature interferons which are specifically described but also any modification of these polypeptides which leave the IFN-omega activity unaffected. These modifications comprise shortening of the molecules at the N- or C-termini thereof, exchanging amino acid residues for other residues without substantially affecting activity, or chemically or biochemically attaching the molecules to other molecules, which may be inert or otherwise. Among the latter can be mentioned hybrid molecules made from one or more omega interferons and/or known α - or β -interferons.

In order to be able to compare the differences between the amino acid and nucleotide sequences of the new interferons, particularly of the omega(Gly) and omega(Glu) interferon, with the amino acid and nucleotide sequences which have already been published for α -interferons and β -interferon (C. Weissmann et al., Phil. Trans. R. Soc. London 299, 7-28 (1982); A. Ullrich et al., J. Molec. Biol. 156, 467-486 (1982);, T. Taniguchi et al., Proc. Nat. Acad. Sci. 77, 4003-4006 (1980); K. Tokodoro

et al., EMBO J. 3, 669-670 (1984)), the corresponding sequences are arranged in pairs and the differences at individual positions are counted.

The results shown in Figure 7 demonstrate that the DNA sequences of the clones P9A2 and E76E9 are related to the sequences of the Type I interferons (e.g. α and δ -interferons). It is also shown that the differences in the amino acid sequences between the individual α -interferons and the new sequences are greater than 41.6% and less than 47.0%. The differences between the new sequences and those of the individual α -interferons and that of δ -interferon are in the order of about 70%. Taking into account the results of Example 4 in which the existence of a whole set of related genes is demonstrated, and also taking into account the proposed nomenclature for interferons (J. Vilceck et al., J. Gen. Virol. 65, 669-670 (1984)) it is assumed that the cDNA inserts of the clones P9A2 and E76E9 code for a new class of Type I interferon which will be referred to as interferon-omega.

It is also shown that the omega-interferon gene expression occurs analogously to that of a Type I interferon gene. Transcription of the individual members of the multi-gene families of the α - and omega-interferons based on the S1 mapping method (A.J. Berk et al., Cell 12, 721 (1977)) is investigated (see Example 7) and it is found that the expression omega-1-mRNA is virus-inducible. Since the transcripts of a gene family of this kind differ by only a few bases out of approximately 1000, hybridisation alone is not a sufficiently sensitive distinguishing feature in order to distinguish between the various IFN mRNA's.

To do this, the mRNA sequences of 9 α -interferons, interferon-omega-1 and δ -interferon are produced. Capital letters are used to designate those bases

which are specific to the top sequence. Such specific sites can easily be found using a simple computer programme. A hybridisation probe which starts from a specific site can only hybridise perfectly with the mRNA of a specific subtype. All other mRNA's are unable to hybridise at the specific site of the subtype. If the hybridisation probe is radioactively labelled at its specific end, only those radioactive labels which are protected from digestion with a single strand-specific nuclease (preferably S1 nuclease) are those which have hybridised with the interferon subtype mRNA for which the probe was designed.

This principle is not restricted to interferon but may be applied to any group of known sequences which have the specific sites described in Figure 8.

The above-mentioned specific sites of the subtypes are not restriction sites, in most cases, which means that the cutting of the cDNA's with restriction endonucleases is not capable of producing subtype-specific hybridisation probes. The probes used in this example were therefore produced by extending an oligonucleotide radioactively labelled at the 5' end which is complementary to the mRNA of interferon-omega above the specific site.

Figure 10 shows that, as expected, omega-1-mRNA can be induced in Namalwa and NC37 cells.

The invention therefore comprises not only the genetic sequences specifically coding for the omega interferons mentioned, but also modifications obtained readily and routinely by mutation, deletion, transposition or addition. Any sequence which codes for human omega-interferons as described (i.e. having a spectrum of biological activities as shown herein) and which is degenerate to those actually shown is also included. Those skilled

in the art understand how to prepare degenerate DNA sequences of the coding regions. Also, any sequence coding for a polypeptide having the spectrum of activities shown herein for IFN-omega, and which hybridises with the sequences (or portions thereof) shown herein under stringent hybridisation conditions (i.e., selecting for better than about 85%, preferably better than about 90% homology) is also covered.

The hybridisations are carried out in 6 x SSC/5 x Denhardt's solution/0.1% SDS at 65°C. The degree of stringency is determined in the washing step. Thus, for a selection to DNA sequences with about 85% homology or more, the conditions 0.2 x SSC/0.01%, SDS/65°C are suitable and for selection to DNA sequences with about 90% homology or more, the conditions 0.1 x SSC/0.01% SDS/65°C are suitable.

When looking through a cosmid library under these conditions (0.2 x SSC) one will find a number of cosmids which hybridise with an IFN-omega probe. Sequence analysis of restriction enzyme fragments isolated therefrom will give the authentic IFN-omega gene (see Figure 11) and three other related genes which have been referred to as IFN-pseudo-omega2, IFN-pseudo-omega3 and IFN-pseudo-omega4 (see Figures 12-14). The invention also relates to these and to the peptides which they code. This is recited in claims 43 to 52.

The DNA comparisons give an approximately 85% homology of the pseudo genes with the IFN-omega gene (interferon-omega-one gene).

Moreover, the IFN-omega gene shows that in transcription the mRNA contains the information for a functional interferon protein, i.e. a signal peptide 23 amino acids long, of the formula

Met Ala Leu Leu Phe Pro Leu Leu
Ala Ala Leu Val Met Thr Ser Tyr Ser Pro Val Gly Ser Leu Gly

is coded, which is followed by the mature IFN-omegal which is 172 amino acids long.

Interferon-omega genes may be introduced into any organism under conditions which result in high yields. Suitable hosts and vectors are well known to anyone skilled in the art; by way of example, reference is made to EP-A-0.093.619.

In particular, prokaryotes are preferred for expression. For example, *E. coli* K12 strain 294 (ATCC No. 31446) is particularly useful. Other microbial strains which may be used include *E. coli* X1776 (ATCC No. 31.537). The aforementioned strains, as well as *E. coli* W3110 (F^- , λ^- , prototrophic, ATCC No. 27325), bacilli such as *Bacillus subtilis*, and other enterobacteria such as *Salmonella typhimurium* or *Serratia marcesens*, and various pseudomonad species may be used.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (Bolivar, et al., Gene 2, 95 (1977)). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid or other plasmids must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins. Those promoters most commonly

used in recombinant DNA construction include the beta-lactamase (penicillinase) and lactose promoter systems (Chang et al., Nature 275, 615 (1978); Itakura et al., Science 198, 1056 (1977); Goeddel et al., Nature 281, 544 (1979)) and tryptophan (trp) promoter system (Goeddel et al., Nucleic Acids Res. 8, 4057 (1980); EP-A-0.036.776).

While these are the most commonly used, other microbial promoters have been discovered and utilized. For example, the genetic sequence for IFN-omega can be placed under the control of the leftward promoter of bacteriophage Lambda (P_L). This promoter is one of the strongest known promoters which can be controlled. Control is exerted by the lambda repressor, and adjacent restriction sites are known.

A temperature sensitive allele of this gene can be placed on the vector that contains the IFN-omega complete sequence. When the temperature is raised to 42°C, the repressor is inactivated, and the promoter will be expressed at its maximum level. The amount of mRNA produced under these conditions should be sufficient to result in a cell which contains about 10% of its newly synthesised RNA originated from the P_L promoter. In this way, it is possible to establish a bank of clones in which a functional IFN-omega sequence is placed adjacent to a ribosome binding sequence, and at varying distances from the lambda P_L promoter. These clones can then be screened and the one giving the highest yield selected.

The expression and translation of an IFN-omega sequence can also be placed under control of other regulons which may be "homologous" to the organism in its untransformed state. For example, lactose dependent E. coli chromosomal DNA comprises a lactose or lac operon which permits lactose digestion by elaborating the enzyme beta-galactosidase.

The lac control elements may be obtained from bacteriophage lambda plac5, which is infective for *E. coli*. The phage's lac operon can be derived by transduction from the same bacterial species. Regulons suitable for use in the process of the invention can be derived from plasmid DNA native to the organism. The lac promoter-operator system can be induced by IPTG.

Other promoter-operator systems or portions thereof can be employed as well: for example, the arabinose-operator, Colicine E₁-operator, galactose-operator, alkaline phosphatase-operator, trp-operator, xylose A-operator, tac-promoter and the like can be used.

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used. Saccharomyces cerevisiae is the most commonly used among eukaryotic microorganisms, although a number of other species are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example (Stinchcomb, et al., Nature 282, 39 (1979); Kingsman et al., Gene 7, 141 (1979); Tschumper, et al., Gene 10, 157 (1980)) and plasmid YEpl3 (Bwach et al., Gene 8, 121-133 (1979)) are commonly used. The plasmid YRp7 contains the TRP1 gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076.

The presence of the TRP1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, the plasmid YEpl3 contains the yeast LEU2 gene which can be used to complement a LEU2 minus mutant strain.

Suitable promoting sequences in yeast vectors include the 5'-flanking region of the genes of ADH I (Ammerer, G., Methods of Enzymology 101, 192-201 (1983)), 3-phosphoglycerate kinase (Hitzeman, et al., J.

Biol. Chem. 255, 2073 (1980)) or other glycolytic enzymes (Kawasaki and Fraenkel, BBRC 108, 1107-1112 (1982)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate, decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, phosphoglucose isomerase, and glucokinase. In concentrating suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector at the 3' end of the sequences to be expressed, to provide polyadenylation of the mRNA and termination.

Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter regions of the genes for alcohol dehydrogenase-2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilisation. Promoters which are regulated by the yeast mating type locus, such as the promoters of the genes BARI, MFa1, STE2, STE3, STE5 can be used for temperature regulated systems by using temperature dependent siv mutations (Rhine, Ph.D. Thesis, University of Oregon, Eugene, Oregon (1979), Herskowitz and Oshima, The Molecular Biology of the Yeast Saccharomyces, part I, 181-209 (1981), Cold Spring Harbor Laboratory). These mutations directly influence the expressions of the silent mating type cassettes of yeast, and therefore indirectly the mating type dependent promoters. Generally, however, any plasmid vector containing a yeast-compatible promoter, originating replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate

or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, Academic Press, Kruse and Patterson, Editors (1973)). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and WI38, BHK, COS-7 and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late end promoters of SV40 are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers et al., Nature 273, 1123 (1978)). Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the Hind III site toward the Bgl I site location in the viral origin of replication. Further, it is also possible, and often desirable, to utilise promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV, etc.) source, or may be provided by the host

cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

The genes may, however, preferably be expressed in an expression plasmid pER103 (E. Rastl-Dworkin et al., Gene 21, 237-248 (1983) and EP-A-0.115-613 - deposited at the DSM under the number DSM 2773 on 20th December 1983), since this vector contains all the regulons which result in a high expression rate of the cloned genes. According to the invention, therefore, the shorter EcoRI/BamHI fragment belonging to the plasmid pBR322 was replaced by the DNA sequence of formula

```

EcoRI      Sau3A
gaattcaccgctGATCGCTAAAACATTGTGCAAAAAGAGGGTTGACTTTGCCTTCGCGA 59

                ↓ mRNA-Start
ACCAGTTAACTAGTACACAAGTTCACGGCAACGGTAAGGAGGCTTTAAGCTTAAAG ATG 116
                                RBS      HindIII

Cys Asp
TGT GAT C — IFN-omega-Gen —→
      Sau3A
  
```

In order to arrive at the objective of the invention, the following procedure is used, for example, according to Figure 6:

I. Preparation of the individual DNA fragments required:

Fragment a

In order to produce fragment a) a plasmid which contains a gene for IFN-omega, e.g. the plasmid P9A2, is digested with the restriction endonuclease AvaII. After chromatography and purification of the resulting cDNA insert, the latter is twice redigested with the restriction endonucleases NcoI

and AluI and then isolated by chromatography and electroelution. This fragment contains the majority of the corresponding omega-interferon gene. Thus, for example, the omega(Gly) interferon gene of the clone P9A2 has the following structure:

```

      5                               10                               15
      His Gly Leu Leu Ser Arg Asn Thr Leu
c | CAT GGC CTA CTT AGC AGG AAC ACC TTG 28
  | NcoI

      20                               25                               30
Val Leu Leu His Gln Met Arg Arg Ile Ser Pro Phe Leu Cys Leu
GTG CTT CTG CAC CAA ATG AGG AGA ATC TCC CCT TTC TTG TGT CTC 73

      35                               40                               45
Lys Asp Arg Arg Asp Phe Arg Phe Pro Gln Glu Met Val Lys Gly
AAG GAC AGA AGA GAC TTC AGG TTC CCC CAG GAG ATG GTA AAA GGG 118

      50                               55                               60
Ser Gln Leu Gln Lys Ala His Val Met Ser Val Leu His Glu Met
AGC CAG TTG CAG AAG GCC CAT GTC ATG TCT GTC CTC CAT GAG ATG 163

      65                               70                               75
Leu Gln Gln Ile Phe Ser Leu Phe His Thr Glu Arg Ser Ser Ala
CTG CAG CAG ATC TTC AGC CTC TTC CAC ACA GAG CGC TCC TCT GCT 208

      80                               85                               90
Ala Trp Asn Met Thr Leu Leu Asp Gln Leu His Thr Gly Leu His
GCC TGG AAC ATG ACC CTC CTA GAC CAA CTC CAC ACT GGA CTT CAT 253

      95                               100                               105
Gln Gln Leu Gln His Leu Glu Thr Cys Leu Leu Gln Val Val Gly
CAG CAA CTG CAA CAC CTG GAG ACC TGC TTG CTG CAG GTA GTG GGA 298

      110                               115                               120
Glu Gly Glu Ser Ala Gly Ala Ile Ser Ser Pro Ala Leu Thr Leu
GAA GGA GAA TCT GCT GGG GCA ATT AGC AGC CCT GCA CTG ACC TTG 343

      125                               130                               135
Arg Arg Tyr Phe Gln Gly Ile Arg Val Tyr Leu Lys Glu Lys Lys
AGG AGG TAC TTC CAG GGA ATC CGT GTC TAC CTG AAA GAG AAG AAA 388

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                140                145                150
Tyr Ser Asp Cys Ala Trp Glu Val Val Arg Met Glu Ile Met Lys
TAC AGC GAC TGT GCC TGG GAA GTT GTC AGA ATG GAA ATC ATG AAA 433

                155                160                165
Ser Leu Phe Leu Ser Thr Asn Met Gln Glu Arg Leu Arg Ser Lys
TCC TTG TTC TTA TCA ACA AAC ATG CAA GAA AGA CTG AGA AGT AAA 478

                170
Asp Arg Asp Leu Gly Ser Ser
GAT AGA GAC CTG GGC TCA TCT TGAAATGATTCTCATTGATTAATTTGCCATA 530

TAACACTTGCACATGTGACTCTGGTCAATTCAAAAGACTCTTATTTTCGGCTTTAATCAC 589
AGAATTGACTGAATTAGTTC TGCAAATAC TTTGTCGGTATATTAAGCCAGTATATGTTA 648
AAAAGACTTAGGTT CAGGGGCATCAGTCCCTAAGATGTTATTTATTTTACTCATTAT 707
TTATTCTTACATTTTATCATATTTATACTATTTATATTC TTATATAACAAATGTTTGCC 766
TTTACATTGTATTAAGATAACAAAACATGTT CAGct 802
                                AluI

```

Fragment b:

In order to produce fragment b) the plasmid P9A2 is digested with the restriction endonuclease *AvaII*. After chromatography and purification of the resulting cDNA insert, the latter is redigested with the restriction endonuclease *Sau3A* and the desired fragment 189bp long is isolated by chromatography and electroelution. It has the following structure:

```

                5                10                15
Asp Leu Pro Gln Asn His Gly Leu Leu Ser Arg Asn Thr Leu
GAT CTG CCT CAG AAC CAT GGC CTA CTT AGC AGG AAC ACC TTG 42
Sau3A|                                NcoI

                20                25                30
Val Leu Leu His Gln Met Arg Arg Ile Ser Pro Phe Leu Cys Leu
GTG CTT CTG CAC CAA ATG AGG AGA ATC TCC CCT TTC TTG TGT CTC 87

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35 40 45
Lys Asp Arg Arg Asp Phe Arg Phe Pro Gln Glu Met Val Lys Gly
AAG GAC AGA AGA GAC TTC AGG TTC CCC CAG GAG ATG GTA AAA GGG 132

50 55 60
Ser Gln Leu Gln Lys Ala His Val Met Ser Val Leu His Glu Met
ACG CAG TTG CAG AAG GGC CAT GTC ATG TCT GTC CTC CAT GAG ATG 177

Leu Gln Gln Ile
CTG CAG CAG atc 189
Sau3A

Fragment c

In order to prepare fragment c) the plasmid pER33 (see E. Rastl-Dworkin et al., Gene 21, 237-248 (1983) and EP-A-0.115.613) is digested twice with the restriction enzymes EcoRI and PvuII. The fragment 389bp long which is obtained after agarose gel fractionation and purification and which contains the Trp promotor, the ribosomal binding site and the starting codon, is subsequently digested with Sau3A. The desired fragment 108bp long is obtained by agarose gel electrophoresis, electroelution and Elutip[®] column purification. It has the following structure:

EcoRI | Sau3A
gaattcacgctGATCGCTAAAACATTGTGCAAAAAGAGGGTTGACTTTGCCTTCGCGA 59
↓ mRNA-Start Met
ACCAGTTAACTAGTACACAAGTTCACGGCAACGGTAAGGAGGTTTAAGCTTAAAG ATG 116
RBS HindIII
Cys Asp
TGT gat c 123
Sau3A

Ligation of fragments b and c:

The fragments b and c are ligated with T4 ligase and, after destruction of the enzyme, cut with HindIII. This ligated fragment has the following structure:

HindIII	Sau3A	NcoI	
a AGCTTAAAG	ATGTGTGATC	TGCCTCAGAA	CCATGGCCTA CTTAGCAGGA 50
ACACCTTGGT	GCTTCTGCAC	CAAATGAGGA	GAATCTCCCC TTTCTTGTGT 100
CTCAAGGACA	GAAGAGACTT	CAGGTTCCCC	CAGGAGATGG TAAAAGGGAG 150
CCAGTTGCAG	AAGGCCCATG	TCATGTCTGT	CCTCCATGAG ATGCTGCAGC 200
AGATCACACA	TCTTTA	agct t	
Sau3A	HindIII		

Alternatively, this DNA fragment necessary for the production of the plasmid pRHW10 may also be produced by using two synthetically produced oligonucleotides:

The oligonucleotide of formula

5'-AGCTTAAAGATGTGT-3'

remains dephosphorylated at its 5' end.

The oligonucleotide of formula

5'-GATCACACATCTTTA-3'

is kinased by means of the T₄ polynucleotide kinase and ATP at the 5' end.

When the two oligonucleotides are hybridised, the following short DNA fragment is obtained:

5'-AGCTTAAAGATGTGT	3'
3'- ATTTCTACACACTAGp	5'

This produces at one end the 5' overlap typical of HindIII and at the other end the 5' overlap typical of Sau3A.

Fragment b) is dephosphorylated using calves' intestine phosphatase. Fragment b) and the fragment described above are combined and joined together by means of T_4 ligase.

Since the ligase requires at least one end containing 5'-phosphate, only the synthetic piece of DNA can be joined to fragment b) or 2 synthetic fragments may be connected at their Sau3A ends. Since the two resulting fragments are of different lengths, they may be separated by selective isopropanol precipitation. The fragment thus purified is phosphorylated using T_4 polynucleotide kinase and ATP.

II. Preparation of the expression plasmids

a) Preparation of plasmid pRHW10:

The expression plasmid pER103 (E. Rastl-Dworkin et al., Gene 21, 237-284 (1983) and EP-A-0.115.613, filed at the DSM under DSM Number 2773) is linearised with HindIII and then treated with calves' intestine phosphatase. After isolation and purification of the DNA thus obtained, it is dephosphorylated and then ligated with ligated fragments b and c (after they have been digested with HindIII). Then E.coli HB 101 is transformed with the resulting ligation mixture and cultivated on LB agar plus 50 µg/ml of ampicillin. The plasmid having the desired structure was designated pRHW 10 (see Figure 6), and after replication was used as an intermediate

for preparing other plasmids.

b) Preparation of the plasmid pRHW12:

The Klenow fragment of the DNA polymerase I and the 4 deoxynucleotide triphosphates are added to the plasmid pRHW10 cut with BamHI. The linearised straight-ended plasmid obtained after incubation is purified and then cut with NcoI. The larger fragment, which is obtained using agarose gel electrophoresis, electroelution and Elutip[®] purification, is ligated with fragment a. Then the ligation mixture is transformed with E. coli HB 101 and cultivated on LB agar plus 50 µg/ml of ampicillin. The plasmid having the structure was designated pRHW12 (see Figure 6) which expresses the desired omega(Gly) interferon.

For example, 1 litre of the incubated bacterial culture thus obtained (optical density: 0.6 at 600 nm) contains 1×10^6 International Units of interferon.

c) Preparation of the plasmid pRHW11:

The Klenow fragment of DNA polymerase I and the 4 deoxynucleotide triphosphates are added to the plasmid pRHW10 cut with BamHI. The linearised straight-ended plasmid obtained after incubation is purified and then cut with NcoI. The larger fragment, which is obtained using agarose gel electrophoresis, electroelution and Elutip[®] purification, is ligated with fragment a obtained analogously from the plasmid E76E9, in which only the GGG codon coding for the amino acid (Gly) is replaced by the GAG codon (Glu). Subsequently the resulting ligation mixture is transformed with E. coli HB101 and cultivated on LB agar. The plasmid having the desired structure was designated pRHW11 (see analogously Figure 6) which expresses the desired

omega(Glu)-interferon.

Transformation of cells with vectors can be effected by a number of procedures. For example, it can be carried out by means of calcium, which either involves washing cells in magnesium and adding DNA to cells suspended in calcium or exposing cells to a coprecipitate of DNA and calcium phosphate. Following gene expression, the cells are plated on media which select for transformants.

After appropriate transformation of the host, expression of the gene therein and fermentation or cell culture under conditions where IFN-omega is expressed, the product can normally be extracted by means of well known chromatographic separation procedures to yield a material comprising IFN-omega with or without leading and trailing sequences. The IFN-omega may be expressed with a leading sequence at the N-terminus thereof (to yield pre-IFN-omega), which may be removed in some of the host cells. If not removed, it may be necessary to cleave the leading polypeptide (if any is present) to yield the mature IFN-omega. Alternatively, the IFN-omega clone can be modified in such a way that the mature protein will be directly produced in the microorganism instead of pre-IFN-omega. In this respect, the precursor sequence of the yeast mating pheromone MF-alpha-1 can be used for precise maturation of the fused protein, and for secretion of the products into the growth medium or periplasmic space. The DNA sequence corresponding to functional or mature IFN-omega can be connected to MF-alpha-1 at the presumed cathepsin-like cleavage site (after lys-arg) at position 256 from the initiation codon ATG (Kurjan, Herskowitz, Cell 30, 933-943 (1982)).

On the basis of their biological actions, the new interferons according to the invention are suitable for the treatment of any conditions

for which the known interferons have been used. These include conditions such as herpes, rhinovirus, opportunistic AIDS infections, certain cancers, and the like. The new interferons can be used by themselves or in combination with other known interferons or other biologically active products, such as IFN-alpha, IL-2, other immune modulators, and the like.

IFN-omega may be parenterally administered to subjects requiring antitumour or antiviral treatment, and to those exhibiting immunosuppressive conditions. Dosage and dose rate may parallel those currently in use in clinical investigations of IFN- α materials, e.g. about $(1-10) \times 10^6$ units daily, and in the case of materials of purity greater than 1%, up to e.g. 5×10^7 units daily.

As one example of an appropriate dosage form for essentially homogenous bacterial IFN-omega in parenteral form, 3 mg IFN-omega may be dissolved in 25 ml of 5 N human serum albumin, the solution is passed through a bacteriological filter and the filtered solution aseptically subdivided into 100 vials, each containing 6×10^6 units pure IFN-omega suitable for parenteral administration. The vials are preferably stored in the cold (-20°C) prior to use.

The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the polypeptide hereof is combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation are described in Remington's Pharmaceutical Sciences by E. W. Martin, to which reference is expressly made. IFN-omega is mixed together with a suitable amount of vehicle in order to prepare pharmaceutically acceptable compositions suitable for effective

administration to the receiver (patient). The preferred mode of administration is parenteral.

The following examples, which are not exhaustive, will illustrate the invention in greater detail.

Example 1

Finding IFN-sequence-specific clones

a) Preparation of a cDNA Library

mRNA from Sendai-virus-stimulated cells was used as initial material for the establishment of a cDNA library according to methods known in the literature (E. Dworkin-Rastl et al., Journal of Interferon Research Vol. 2/4, 575-585 (1982)). The 30,000 clones obtained were individually transferred into the wells of microtiter plates. The following medium was used for growing and freezing the colonies:

10	g Trypton
5	g Yeast Extract
5	g NaCl
0.51	g Na-Citrate x 2 H ₂ O
7.5	g K ₂ HPO ₄ x 2 H ₂ O
1.8	g KH ₂ PO ₄
0.09	g MgSO ₄ x 7 H ₂ O
0.9	g (NH ₄) ₂ SO ₄
44	g glycerine
0.01	g Tetracycline x HCl
<u>ad</u>	1 l H ₂ O

The microtiter plates with the individual clones were incubated overnight at 37°C and were then stored at -70°C.

b) Hybridisation Test

As the starting material for the hybridisation test was used the recombinant plasmid pER33 (E. Dworkin-Rastl et. al., Gene 21, 237-248 (1983)). This plasmid contains the coding region for the mature interferon IFN-α2 arg plus 190 bases of the 3' nontranslated region. 20 µg pER33 were incubated with 30 units of Hind III restriction endonuclease in 200 µl reaction solution (10 mM Tris-HCl, pH-7.5, 10 mM MgCl₂, 1 mM Dithiotreitol (DTT), 50 mM NaCl) for 1 hour at 37°C. The reaction

was terminated by the addition of 1/25 vol 0.5 M ethylenedinitrilotetraacetic acid (EDTA) and heating to a temperature of 70°C for 10 minutes. After the addition of 1/4 vol 5 x buffer (80% glycerine, 40 mM Tris acetate, pH 7.8, 50 mM EDTA, 0.05% Sodium dodecylsulphate (SDS), 0.1% bromophenol blue), the developing fragments were separated electrophoretically according to size in a 1% agarose gel. [Gel and electrophoresis buffer (TBE): 10.8 g/l trishydroxymethylaminomethane (Tris-Base), 5.5 g/l boric acid, 0.93 g/l EDTA]. After the incubation of the gel in a 0.5 µg/ml ethidium bromide solution, the DNA strips were made visible in UV-light and the gel area, which contained the IFN-gene-containing DNA piece (about 800 bp long), was cut. By applying a voltage, the DNA was electroeluted into 1/10 x TBE buffer. The DNA solution was extracted once with phenol and four times with ether, and the DNA was precipitated by adding 1/10 vol 3 M sodium acetate (NaAc) pH 5.8 and 2.5 vol EtOH from the aqueous solution at -20°C. After centrifuging, the DNA was washed with 70% ethanol and was dried in a vacuum for 5 minutes. The DNA was placed in 50 µl of water (about 50 µg/µl). The DNA was marked radioactively by means of nick translation (modified according to T. Maniatis et al., Molecular Cloning, Ed. CSH). Furthermore, 50 µl incubation solution contained the following:

50 mM Tris pH 7.8, 5 mM MgCl₂, 10 mM mercaptoethanol, 100 ng DNA insert from pER33, 16 pg DNaseI, 25 µM dATP, 25 µM dGTP, 25 µM dTTP, 20 µCi α -³²P-dCTP (>3,000 Ci/mMol), as well as 3 units of DNA polymerase I (E.coli). Incubation was performed at 14°C for 45 minutes. The reaction was terminated through the addition of 1 vol 50 mM EDTA, 2% SDS, 10 mM Tris pH=7.6 solution and heating to 70°C for 10 minutes. The DNA was separated by means of chromatography

using Sephadex [®]G-100 into TE buffer (10 mM Tris pH=8.0,

1 mM EDTA) from radioactivity that was not internal. The radioactively marked sample had a specific radioactivity of about 4×10^7 cpm/ μ g.

c) Screening the Clones for IFN-gene-containing inserts

The bacterial cultures, which had been frozen into the microtiter plates, were thawed (a). A piece of nitrocellulose filter of corresponding size (Schleicher and Schüll, BA 85, 0.45 μ m pore size) was placed on LB-agar (LB-agar: 10 g/l Trypton, 5 g/l yeast extract, 5 g/l NaCl, 15 g/l Bacto Agar, 20 mg/l Tetracycline-HCl). By means of a plunger adapted to the microtiter plates, the individual clones were transferred to the nitrocellulose filter. The bacteria grew overnight at 37°C to form colonies with a diameter of about 5 mm. To destroy the bacteria and to denature the DNA, the nitrocellulose filters were, one after the other, placed on a stack of Whatman 3MM Filter which had been soaked with the following solutions: (1) 8 minutes at 0.5 M NaOH, (2) 2 minutes at 1 M Tris pH=7.4, (3) 2 minutes at 1 M Tris pH=7.4 and (4) 4 minutes at 1.5 M NaCl, 0.5 M Tris pH=7.4. The filters were dried in air and were then kept at 80°C for 2 hours. The filters were pretreated for 4 hours at 65°C in the hybridisation solution, consisting of 6 x SSC (1 x SSC corresponds to 0.15 M NaCl; 0.015 M trisodium citrate; pH = 7.0), 5 x Denhardt's solution (1 x Denhardt's solution corresponds to 0.02% PVP (polyvinylpyrrolidone); 0.02% ficoll (MG: 40,000D); 0.02% BSM (Bovine Serum Albumin) and 0.1% SDS (sodium dodecylsulphate). About 1×10^6 cpm per filter of the sample made in (b) were denatured by boiling and added to the hybridisation solution. Hybridisation was performed at 65°C for a period of 16 hours. The filters were washed four times

for 1 hour at 65°C with 3 x SSC/0.1% SDS. The filters were dried in air, were covered with Saran Wrap[®], and exposed on Kodak X-OmatS[®] film.

Example 2

Southern Transfer to Confirm IFN-gene-containing Recombinant Plasmids

Of the positively reacting colonies or those colonies that were suspected of reacting positively, 5 ml cultures were produced in L-broth (10 g/l Trypton, 5 g/l Yeast Extract, 5 g/l NaCl, 20 mg/l Tetracycline x HCl) at 37°C overnight. The plasmid-DNA was isolated in a modified manner according to Birnboim and Doly (Nucl. Acid. Res. 7, 1513 (1979)). The cells in 1.5 ml suspension were centrifuged (Eppendorf Centrifuge) and resuspended at 0°C in 100 µl lysozyme solution consisting of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH=8.0 and 4 mg/ml of lysozyme. After 5 minutes of incubation at room temperature, 2 vol of ice-cold 0.2 M NaOH, 1% SDS solution were added, and incubation continued for another 5 minutes. Then 150 µl of ice-cold sodium acetate solution pH=4.8 was added and incubated for 5 minutes. The precipitated cell components were centrifuged. The DNA solution was extracted with 1 vol phenol/CHCl₃ (1:1), and the DNA was precipitated by the addition of 2 vol ethanol. After centrifuging, the pellet was washed once with 70% ethanol, and dried in a vacuum for 5 minutes. The DNA was dissolved in 50 µl (TE)-buffer. Of that amount, 10 µl were digested in 50 µl reaction solution (10 mM Tris-HCl pH=7.5, 10 mM MgCl₂, 50 mM NaCl, 1 mM DTT) with 10 units PstI-restriction endonuclease for 1 hour at 37°C. After the addition of 1/25 vol 0.5 M EDTA as well as 1/4 vol 5 x buffer (see Example 1b)), it was heated for 10 minutes and the DNA was then separated electrophoretically

in a 1% agarose gel (TBE-buffer). The DNA in the agarose gel was transferred to a nitrocellulose filter according to the method of Southern (E. M. Southern, J. Mol. Biol. 98, 503-517 (1975)). The DNA in the gel was denatured by means of 1 hour incubation of the gel with a 1.5 M NaCl/0.5 M NaOH solution. This was followed by neutralisation for 1 hour with a 1 M Tris x HCl pH=8/1.5 M NaCl solution. The DNA was transferred to the nitrocellulose filter with 10 x SSC (1.5 M NaCl, 0.15 M sodium citrate, pH=7.0). After completion of transfer (about 16 hours), the filter was briefly rinsed in 6 x SSC buffer and then dried in air; it was finally baked at 80°C for 2 hours. The filter was pretreated for 4 hours with a 6 x SSC/5 x Denhardt's solution / 0.1% SDS (see Example 1c) at 65°C. About 2×10^6 cpm of the hybridisation sample (see Example 1b) were denatured by means of heating to a temperature of 100°C and were then added to the hybridisation solution. The duration of hybridisation was 16 hours at 65°C. Then the filter was washed 4 x 1 hours at 65°C with a 3 x SSC/0.1% SDS solution. After air-drying, the filter was covered with Saran Wrap[®] and was exposed on Kodak X-OmatS[®] film.

Example 3

Detection of Interferon Activity in the Clone E76E9

A 100-ml culture of clone E76E9 was cultured in L-broth (10 g/l Trypton, 5 g/l yeast extract, 5 g/l NaCl, 5 g/l glucose, 20 mg per l tetracycline x HCl) at 37°C up to an optical density of $A_{600}=0.8$. The bacteria were centrifuged for 10 minutes at 7,000 rpm, they were washed once with a 50 mM Tris x HCl pH=8.0, 30 mM NaCl solution and then were resuspended in 1.5 ml washing solution. After incubation with 1 mg/ml of lysozyme at 0°C for

half an hour, the bacterial suspension was frozen and thawed five times. The cells were pelletized by means of centrifugation at 40,000 rpm for 1 hour. The supernatant was sterile filtered and was tested for interferon activity. The test used was the Plaque Reduction Test with V3 cells and Vesicular Stomatitis Virus (G. R. Adolf et al., Arch. Virol. 72, 169-178 (1982)). Surprisingly, the clone produced up to 9,000 IU of interferon per litre of initial culture.

Example 4

Genomic Southern Blot for determining the number of genes associated with the new sequences

a) Isolating the DNA from Namalwa cells

400 ml of a Namalwa cell culture are centrifuged at 1000 rpm in a JA21 centrifuge in order to pelletise the cells. The resulting pellets are carefully washed by resuspending them in NP40 buffer (NP40 buffer: 140 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris/Cl pH=7.4) and pelletising them again at 1000 rpm. The pellets obtained are again suspended in 20 ml of NP40[®] buffer and mixed with 1 ml of a 10% NP40[®] solution in order to destroy the cell walls. After standing for 5 minutes in an ice bath the intact cell nuclei are pelletised by centrifuging at 1000 rpm and the supernatant is discarded. The cell nuclei are resuspended in a 10 ml of a solution consisting of 50 mM Tris/Cl pH=8.0, 10 mM EDTA and 200 mM NaCl, and then 1 ml of 20% SDS are added to eliminate the proteins. The resulting viscous solution is extracted twice with the same quantity of phenol (saturated with 10 mM Tris/Cl pH=8.0) and twice with chloroform. The DNA is precipitated by adding ethanol, separated off by centrifuging, then the resulting DNA pellet is washed once with 70% ethanol, dried for 5 minutes in vacuo and dissolved in 6 ml of TE buffer (TE buffer: 10 mM

Tris/Cl pH=8.0, 1 mM EDTA). The concentration of the DNA is 0.8 mg/ml.

b) Restriction endonuclease digestion of the DNA from Namalwa cells

The restriction endonuclease digestion was carried out in accordance with the conditions specified by the manufacturer (New England Biolabs). 1 µg of DNA is digested with 2 units of the suitable restriction endonuclease in a volume of 10 µl at 37°C for 2 hours or longer. EcoRI, HindIII, BamHI, SphI, PstI and ClaI are used as restriction endonucleases. 20 µg of DNA are used for each digestion. In order to monitor that the digestion is complete, 10 µl (aliquot parts) are separated off at the start of the reaction and mixed with 0.4 µg of lambda phage-DNA. After 2 hours incubation, these aliquot portions are monitored by agarose gel electrophoresis and the completeness of digestion is assessed with the aid of a sample of the coloured lambda phase DNA fragments.

After these checks have been carried out the reactions are stopped by adding EDTA to a final concentration of 20 mM and heating the solution to 70°C for 10 minutes. The DNA is precipitated by the addition of 0.3 M NaAc, pH=5.6 and 2.5 vols of ethanol. After 30 minutes incubation at -70°C the DNA is pelletised in an Eppendorf centrifuge, washed once with 70% ethanol and dried. The resulting DNA is taken up in 30 µl of TE buffer.

c) Gel electrophoresis and Southern Transfer

The digested DNA samples are fractionated according to their size in a 0.8% agar gel in TBE buffer (10.8 g/l Tris base, 5.5 g/l boric acid, 0.93 g/l EDTA). For this purpose, 15 µl of the DNA sample are mixed with 4 µl of charging buffer

(0.02% SDS, 5 x TBE buffer, 50 mM EDTA, 50% glycerine, 0.1% bromophenol blue), heated briefly to 70°C and introduced into the troughs provided in the gel. Lambda-DNA which has been cut with EcoRI and HindIII is introduced into a corresponding trough and serves as a marker for the size of the DNA. Gel electrophoresis is carried out for 24 hours at about 1 V/cm. Then the DNA is transferred to a nitrocellulose filter using the method of Southern (Schleicher and Schuell, BA85), using as the transfer buffer 10 x SSC (1 x SSC: 150 mM trisodium citrate, 15 mM NaCl, pH=7.0). After the filter has been dried at ambient temperature it is heated for 2 hours to 80°C in order to bind the DNA to it.

d) Hybridisation probe

20 µg of the plasmid P9A2 are cut with AvaII, thereby liberating a fragment approximately 1100 bp long which contains the entire cDNA insert. This DNA fragment is again cut with Sau3A and AluI and the largest DNA fragment is isolated after agarose gel electrophoresis (see Figure 5b) by electroelution and Elutip[®] column chromatography. The resulting DNA (1.5 µg) is dissolved in 15 µl of water.

20 µg of the plasmid pER33 are cut with HindIII, and this expression plasmid for IFN-α2-Arg (E. Rastl.-Dworkin et al. Gene 21, 237-248 (1983)) is cut twice. The smaller DNA fragment contains the gene for interferon-α2-Arg and is isolated in the same way as in the case of the plasmid P9A2.

Both DNA's are nick-translated using the method proposed by P.W.J. Rigby et al. (J. Mol. Biol. 113, 237-251 (1977)). The nick translation is carried out with 0.2 µg of DNA in a solution of 50 µl, consisting of 1 x nick buffer (1 x nick buffer: 50 mM Tris/Cl pH=7.2, 10 mM MgSO₄, 0.1 mM

DTT, 50 µg/ml BSA), 100 µmol each of dATP, dGTP and dTTP, 150 µCi α -³²P-dCTP (Amersham, 3000 Ci/mMol) and 5 units of DNA polymerase I (Boehringer-Mannheim, nick translation quality). After 2 hours at 14°C the reaction is stopped by adding the same amount of an EDTA solution (40 mMol) and the unreacted radioactive material is separated off by means of Sephadex® G50 column chromatography in TE buffer. The remaining specific radioactivity is approximately 100×10^6 cpm/µg DNA.

e) Hybridisation and autoradiography

The nitrocellulose filter is cut into two halves. Each half contains an identical set of traces with Namalwa DNA which have been treated with the restriction enzymes mentioned in Example 4a. The filters are pre-hybridised in a solution containing 6 x SSC, 5 x Denhardt's (1 x Denhardt's: 0.02% bovine serum albumin (BSA), 0.02% polyvinylpyrrolidone (PVP), 0.02% Ficoll 400), 0.5% SDS, 0.1 mg/ml of denatured calf thymus DNA and 10 mM EDTA for 2 hours at 65°C. Hybridisation is carried out in a solution containing 6 x SSC, 5 x Denhardt's, 10 mM EDTA, 0.5% SDS and approximately 10×10^6 cpm of nick translated DNA for 16 hours at 65°C. One half of the filter is hybridised with interferon- α 2-Arg-DNA and the other half with interferon-DNA which has been isolated from the plasmid P9A2. After hybridisation, both filters are washed at ambient temperature, four times with a solution consisting of 2 x SSC and 0.1% SDS and twice at 65°C for 45 minutes with a solution consisting of 0.2 x SSC and 0.01% SDS. The filters are then dried and exposed to a Kodak X-Omat® S film.

Example 5

Preparation of the expression plasmids pRHW 12
and pRHW 11

Preliminary comment:

The preparation of the expression plasmids is illustrated in Figure 6 (not to scale), and also all the restriction enzyme digestions are carried out in accordance with the instructions of the enzyme manufacturers.

a) Preparation of the plasmid pRHW 10

100 µg of the plasmid P9A2 are digested with 100 units of the restriction endonuclease *Ava*II (New England Biolabs). After digestion, the enzyme is deactivated by heating to 70°C and the fragments obtained are fractionated on a 1.4% agarose gel with TBE buffer (TBE buffer: 10.8 g/l Tris base, 5.5 g/l boric acid, 0.93 g/l EDTA) according to their size. The band which contains the entire cDNA insert is electroeluted and purified using an Elutip[®] column (Schleicher & Schuell). Of the 20 µg obtained, 6 µg are further digested with the restriction endonuclease *Sau*3a (20 units in a total of 100 µl of solution). The fragments are separated using 2% agarose gel in TBE buffer. After staining with ethidium bromide (EtBr) the DNA fragment 189 bp long is electroeluted and purified as described above (= fragment b in Figure 6).

In order to link the interferon gene with a promoter, a ribosomal binding site and a starting codon, the corresponding DNA fragment is isolated from the expression plasmid pER 33 (E. Rastl-Dworkin et al., Gene 21, 237-248 (1983)). For this purpose 50 µg of pER 33 are digested twice with the restriction enzymes *Eco*RI and *Pvu*II and the resulting fragments are fractionated according to their size on a 1.4% agarose gel in TBE buffer. The DNA fragment which

is 389 bp long and contains the Trp promotor, the ribosomal binding site and the starting codon, is electroeluted and purified using an Elutip[®] column. The fragment thus obtained is then digested with Sau3A and the desired fragment 108 bp is obtained by agarose gel electrophoresis, electroelution and elutip column purification in a yield of approximately 100 ng (= fragment c in Figure 6).

20 ng of fragment b are ligated with 20 ng of fragment c in a volume of 40 µl using 10 units of T4 ligase in a solution containing 50 mM Tris/Cl pH=7.5, 10 mM MgCl₂, 1 mM DTT and 1 mM ATP, at 14°C for 18 hours. The enzyme is then destroyed by heating to 70°C and the resulting DNA is cut with HindIII in a total volume of 50 µl.

10 µg of the expression plasmid pER 103 (E. Rastl-Dworkin et al., Gene 21, 237-248 (1983)) is linearised with HindIII in a total volume of 100 µl. After 2 hours at 37°C 1 volume of 2 x phosphatase buffer (20 mM Tris/Cl pH=9.2, 0.2 mM EDTA) together with one unit of calves intestine phosphatase (CIP) are added. After 30 minutes at 45°C, a further unit of CIP is added and the incubation is continued for 30 minutes. The DNA thus obtained is purified by extracting twice with phenol, once with chloroform and precipitating by the addition of 0.3 M sodium acetate (pH=5.5) and 2.5 Vol ethanol. It is then dephosphorylated in order to prevent religation of the vector during the next ligation step.

100 ng of the linearised pER 103 and the ligated fragment b and c (after HindIII digestion) are added to a solution of 100 µl which contains ligase buffer and ligated with 18 hours at 14°C.

200 µl of competent E. coli HB 101 (E. Dworkin et al., Dev. Biol. 76, 435-448 (1980)) are mixed with 20 µl of the ligation mixture and incubated for 45 minutes on ice. Then absorption of DNA

is completed by a heat shock lasting 2 minutes at 40°C. The cell suspension is incubated for a further 10 minutes on ice and finally applied to LB agar (10 g/l trypton, 5 g/l yeast extract, 5 g/l NaCl, 1.5% agar), containing 50 mg/l ampicillin. The plasmids contained in the 24 colonies obtained are isolated using the method of Birnboim and Doly (see Nucl. Acid. Res. 7, 1513-1523 (1979)). After digestion with various restriction enzymes one plasmid has the desired structure. This is designated PRHW 10 (see Figure 6).

b) Preparation of the plasmid pRHW 12

About 10 µg of the plasmid pRHW 10 are cut with BamHI. Then the Klenow fragment of DNA polymerase I and the 4 deoxynucleoside triphosphates are added and incubated for 20 minutes at ambient temperature. The linearised and straight-ended plasmid obtained is purified by phenol extraction and precipitation and then cut with the restriction endonuclease NcoI in a volume of 100 µl. The larger fragment is obtained by agarose gel electrophoresis, electroelution and elutip purification. The fragment a) (see Figure 6) is obtained by digestion of 4 µg of AvaII fragment which contains the P9A2-cDNA insert (see above) with NcoI and AluI, thereby obtaining about 2 µg of fragment a).

In the final ligation step, fragment a) and the pRHW 10 added thereto, which has twice been digested with BamHI/NcoI, is ligated in a volume of 10 µl, using 10 ng of each DNA. Ligation of a filled BamHI cutting site to a DNA cut by AluI again produces a BamHI cutting site.

The resulting ligation mixture is transformed with competent E. coli HB 101 as described above. Of the 40 colonies obtained, one is selected; this is designated pRHW 12.

The plasmid is isolated and the EcoRI/BamHI insert is sequenced using the method of Sanger (F. Sanger et al., Proc. Nat. Acad. Sci 74, 5463-5467 (1979)). This has the expected sequence.

c) Preparation of the plasmid pRHW 11

This is carried out analogously to Example 5b. 1 µg of the plasmid pRHW 10 is digested with BamHI. The sticky ends of the resulting DNA are blunted using the Klenow fragment of the DNA polymerase I and the 4 deoxynucleoside triphosphates and then the linearised DNA is cut with NcoI. The larger fragment is obtained by agarose gel electrophoresis, electroelution and Elutip[®] column chromatography.

The NcoI-AluI fragment is isolated from the clone E76E9 analogously to Example 5b. Then 10 ng of the vector part is ligated with 10 ng of the cDNA part in a volume of 10 µl under suitable conditions using 1 unit of T4 ligase. After transformation of the resulting DNA mixture in E. coli HB 101 and selection of the 45 resulting colonies on LB plates containing ampicillin, a clone is selected, which is designated pRHW 11. After the corresponding clone has been cultivated, the plasmid DNA is isolated. Its structure is proved by the presence of several specific restriction endonuclease cutting sites (AluI, EcoRI, HindIII, NcoI, PstI).

d) Expression of the interferon activity by E. coli HB 101 containing the plasmid pRHW 12

100 ml of the bacterial culture are incubated up to an optical density of 0.6 at 600 nm in M9 minimal medium which contains all the amino acids with the exception of tryptophan (20 µg/ml per amino acid), 1 µg/ml of thiamine, 0.2% glucose and 20 µg/ml of indol-(3)-acrylic acid (IAA), the inductor of the tryptophan operon. Then the

bacteria are pelletised by centrifuging (10 minutes at 7000 rpm), washed once with 50 mM Tris/Cl pH=8, 30 mM NaCl and finally suspended in 1.5 ml of the same buffer. After 30 minutes incubation with 1 mg/ml of lysozyme on ice the bacteria are frozen and thawed five times. The cell remains are eliminated by centrifuging for 1 hour at 40,000 rpm. The supernatant is filtered sterile and tested for interferon activity in a plaque reduction assay using human A549 cells and encophalo-myocarditis virus.

Result: 1 litre of the bacterial culture produced contains 1×10^6 international units of interferon (A. Billiau, Antiviral Res. 4, 75-98 (1984)).

Example 6

Summary of the differences between the amino acid and nucleotide sequences of Type I interferons

a) Comparison of the amino acid sequences

A comparison of amino acid sequences by pairs is obtained by aligning the first cysteine residue of mature α -interferon with the first cysteine residue of the amino acid sequences which are coded by the cDNA inserts of the plasmids P9A2 and E76E9. The two sequences are shown in Figure 7 as IFN- ω , since no differences could be detected between the specific sequences of the P9A2 or E76E9 clones in the values obtained. The only correction made was the insertion of a gap at position 45 of the interferon- α A, which was counted as an error. If the sequence of the omega-interferon is a partner, the comparison is carried out taking into account the usual 166 amino acids. This value is shown in Figure 7 together with the additional 6 amino acids coded by the clones P9A2 and E76E9. The percentage differences are obtained by dividing the differences by the number 1.66. An additional

amino acid thus represents a percentage of 0.6. This gives 3.6% for the 6 additional amino acids of IFN-omega which are already contained in the percentage.

The comparisons with δ -interferon are carried out by aligning the 3rd amino acid of the mature δ -interferon with the first amino acid of the mature α -interferon or the first amino acid which is coded by the plasmids P9A2 and E76E9. The longest comparison structure of an α -interferon with δ -interferon is thus over 162 amino acids, which gives 2 additional amino acids each for the α -interferon and δ -interferon. These are counted as errors and are shown separately in Figure 7 but they are included in the percentage. The listing of δ -interferon with the amino acid sequences of the clones P9A2 or E76E9 is carried out in the same way. However, this gives a total of 10 additional amino acids.

b) Comparison of the nucleotide sequences

The sequences which are to be compared are listed analogously to Example 6b. The first nucleotide of the DNA of the mature α -interferon is the first nucleotide of the triplet of mature α -interferon coding for cysteine. The first nucleotide from the DNA of the plasmids P9A2 or E76E9 is also the first nucleotide of the codon for cysteine-1. The first nucleotide from the DNA of δ -interferon is the first nucleotide of the third triplet. The comparison is made over a total of 498 nucleotides if the individual DNA's of the α -interferons are compared with the DNA of δ -interferon, and over 516 nucleotides if the DNA sequences of the individual α -interferons or of the δ -interferon are compared with those of the plasmids P9A2 and E76E9. The absolute number of gaps is given in the left-hand part of the Table in Figure 7 and then the corresponding

percentages are given in brackets.

Example 7

Virus-inducible expression of omega-1-mRNA and NC37 cells

a) Synthesis of an omega-interferon specific hybridisation probe

10 pMol of the oligonucleotide d(TGCAGGGCTGCTAA) are mixed with 12 pMol of gamma-³²P-ATP (specific activity: > 5000 Ci/mMol) and 10 units of polynucleotide kinase in a total volume of 10 µl (70 mM Tris/Cl pH = 7.6, 10 mM MgCl₂, 50 mM DTT) and left to stand for one hour at 37°C. The reaction is then stopped by heating to 70°C for 10 minutes. The resulting radioactively labelled oligonucleotide is hybridised with 5 pMol of M13pRHW 12 ssDNA (see Figure 9) in a total volume of 35 µl (100 mM NaCl) by standing for one hour at 50°C.

After cooling to ambient temperature, nick translation buffer, the 4 deoxynucleoside triphosphates and 10 units of Klenow polymerase are added to give a total volume of 50 µl (50 mM Tris/Cl pH = 7.2, 10 mM MgCl₂, 50 µg/ml BSA, 1 mM per nucleotide). Polymerisation is carried out by standing for one hour at ambient temperature and then the reaction is stopped by heating to 70°C for 5 minutes.

In the reaction, a partially double-stranded circular DNA is obtained. This is then cut in a total volume of 500 µl with 25 units of AvaII, using the buffer described by the manufacturer. The double stranded regions are cut to uniform sizes. The reaction is then stopped by heating to 70°C for 5 minutes.

b) Preparation of RNA from virus-infected cells

100 x 10⁶ cells (0.5 x 10⁶/ml) are treated with 100 µMol of dexamethasone for 48 to 72 hours

- the control contains no dexamethasone. To induce interferon, the expression cells are suspended in serum-free medium in a concentration of 5×10^6 /ml and infected with 2^{10} units/ml of Sendai virus. Aliquot parts of the cell culture supernatants are tested for IFN activity in a plaque reduction assay (Example 5b). The cells are harvested 6 hours after the virus infection by centrifuging (1000 g, 10 minutes), washed in 50 ml of NP40[®] buffer (Example 4a), resuspended in 9.5 ml of ice cold NP40[®] buffer and lysed by the addition of 0.5 ml of 10% NP40[®] for 5 minutes on ice. After the nuclei have been removed by centrifuging (1000 x g, 10 minutes) the supernatant is adjusted to pH=8 with 50 mM Tris/Cl, 0.5% Sarcosine[®] and 5 mM EDTA and then stored at -20°C. In order to isolate the total RNA from the supernatant, it is extracted once with phenol, once with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol. The aqueous phase is transferred to a 4 ml 5.7 molar CsCl pad and centrifuged in an SW40 rotor (35 krpm, 20 hours) in order to free the extract from DNA and remaining proteins. The resulting RNA pellet is resuspended in 2 ml of TE, pH=8.0, and precipitated with ethanol. The RNA precipitated is then dissolved in water at a concentration of 5 mg/ml.

c) Detection of interferon-omega mRNA

0.2 µl of the hybridisation probe prepared in Example 7b are precipitated together with 20-50 µg of the RNA prepared according to Example 7c by the addition of ethanol. In the control experiment, instead of the cellular RNA, transfer RNA (tRNA) or RNA originating from E. coli transformed with the plasmid pRHW 12 (Example 5) is added. The resulting pellets are washed free from salt with

70% ethanol, dried and dissolved in 25 µl of 80% formamide (100 mM PIPES pH=6.8, 400 mM NaCl, 10 mM EDTA). Then the samples are heated to 100°C for 5 minutes in order to denature the hybridisation sample, adjusted directly to 52°C and incubated for 24 hours at this temperature. After hybridisation, the samples are placed on ice and 475 µl of S1 reaction mixture (4 mM Zn(Ac)₂, 30 mM NaAc, 250 mM NaCl, 5% glycerine, 20 µg ss calf thymus DNA, 100 units S1 nuclease) are added. After digestion at 37°C for 1 hour the reaction is stopped by ethanol precipitation.

The pellets are dissolved in 6 µl formamide buffer and separated essentially like samples from DNA sequencing reactions on a 6% acrylamide gel containing 8 M urea (F. Sanger et al., Proc. Nat. Acad. Sci. 74, 5463-5467 (1979)).

For autoradiography, the dried gel is exposed to a DuPont Cronex[®] X-ray film using the Kodak Lanex Regular[®] intensivator at -70°C.

Legend relating to Figure 10

Traces A to C represent the controls.

Trace A: 20 µg tRNA

Trace B: 10 µg RNA from pER 33 (E. coli - expression strain for interferon-α2-Arg)

Trace C: 1 ng RNA from pRHW 12 (E. coli expression strain for interferon-omega 1)

Trace D: 50 µg RNA from untreated Namalwa cells

Trace E: 50 µg RNA from virus-infected Namalwa cells

Trace F: 50 µg RNA from Namalwa cells pretreated with dexamethasone and infected with virus

Trace G: 20 µg of RNA from untreated NC 37 cells.

Trace H: 20 µg RNA from virus-infected NC 37 cells

Trace I: 20 µg RNA from NC 37 cells pretreated

with dexamethasone and infected with virus

Trace M: Size marking (pBR 322 cut with HinfI):

Traces B and C show that the expected signal can only be detected when an omegal-specific RNA is among the RNA molecules. They also show that even a large excess of the wrong RNA does not cause a background signal (see trace B). Furthermore, the tRNA used as a hybridisation partner does not produce a signal either (see trace A).

Traces G to I show the induction of the omegal-specific RNA in virus-infected NC 37 cells. The pretreatment with dexamethasone reinforces this effect.

Traces D to F show fundamentally the same result as is obtained with Namalwa cells. However, the induction with omegal-specific RNA is not as great as in the NC 37 cells. This result is parallel to the interferon titres which were measured in the associated cell supernatant.

This behaviour of interferon-omegal gene expression is thus as would be expected from an interferon Type I gene.

Example 8

Isolation of the gene coding for IFN-omegal or genes related thereto:

a) Cosmid screening

A human cosmid bank (human DNA (male) cloned in the cosmid vector pcos2 EMBL (A. Ponstka, H.-R. Rockwitz, A.-M. Frischauf, B. Hohn, H. Lehrach Proc. Natl. Acad. Sci. 81, 4129-4133 (1984)) with a complexity of 2×10^6) was searched for IFN-omegal or related genes. E. coli DH1 (r_K^- , m_K^+ , rec.A; gyrA96, sup.E) was used as the host. First of all, Mg^{++} cells ("plating bacteria") were prepared.

E. coli DH1 grows overnight in L broth (10 g/l trypton, 5 g/l yeast extract, 5 g/l NaCl) supplemented with 0.2% maltose. The bacteria are removed by centrifuging and taken up in a 10 mM MgSO_4 solution to give an optical density₆₀₀ = 2. 5 ml of this cell suspension are incubated with 12.5×10^6 colony forming units of packed cosmids for 20 minutes at 37°C. Then 10 vol of LB are added and the suspension is kept at 37°C for one hour for the purpose of expression of the kanamycin resistance imparted by the cosmid. The bacteria are then removed by centrifuging, resuspended in 5 ml of LB and spread over nitrocellulose filter in 200 μl aliquots (BA85, Schleicher and Schüll, 132 mm diameter) lying on LB agar (1.5% agar in L broth) plus 30 $\mu\text{g/ml}$ kanamycin. About 10,000 to 20,000 colonies grow on each filter. The colonies are replica-plated on further nitrocellulose filters which are kept at 4°C.

A set of the colony filters is processed as described in Example 1c), i.e. the bacteria are denatured, and the single strand DNA is fixed to the nitrocellulose. The filters are washed for 4 hours at 65°C in a 50 mM Tris/HCl, pH=8.0, 1 M NaCl, 1 mM EDTA, 0.1% SDS solution. The filters are then incubated at 65°C for 2 hours in a 5 x Denhardt's (see Example 1c), 6 x SSC, 0.1% SDS solution and hybridised with about 50×10^6 cpm of nick-translated denatured IFN- ω gal DNA (HindIII-BamHI insert of the clone pRHW12, see Figure 6) for 24 hours at 65°C in the same solution. After hybridisation, the filters are washed first 3 x 10 minutes at ambient temperature in a 2 x SSC, 0.01% SDS solution and then 3 x 45 minutes at 65°C in a 0.2 x SSC, 0.01% SDS solution. The filters are dried and exposed to Kodak X-Omat S film using an intensifier film at -70°C. Positively reacting colonies are localised on the replica filters, scratched off

and resuspended in L broth + kanamycin (30 µg/ml). Of this suspension, a few µl are spread out on LB agar + 30 µg/ml kanamycin. The resulting colonies are replica-plated on nitrocellulose filters. These filters are hybridised with ³²P-marked IFN-omegal-DNA as described above. From each hybridising colony, the cosmid is isolated using the method described by Birnboim & Doly (Nucl. Acids Res. 7, 1513 (1979)). With this cosmid DNA preparation, E. coli DH1 was transformed and the transformants were selected on LB agar + 30 µg/ml kanamycin. The transformants were again tested with ³²P-radioactively labelled IFN-omegal DNA for positively reacting clones. One clone in each case starting from the original material isolated is selected and the cosmid thereof is produced on a larger scale (Clewell, D.B. and Helinski, D.R., Biochemistry 9, 4428 (1970)). Three of the isolated cosmids carry the names cos9, cos10 and cosB.

b) Sub-cloning of hybridising fragments in PUC8

1 µg of Cosmids cos9, cos10 and cosB were cut with HindIII under the conditions recommended by the manufacturer (New England Biolabs). The fragments are separated on 1% agarose gels in TBE buffer by electrophoresis and transferred to nitrocellulose filters according to Southern (Example 4c). The two filters are hybridised with nick-translated omegal DNA as described in Example 4d, and washed and exposed. About 20 µg of each cosmid are cut with HindIII and the fragments formed are separated by gel electrophoresis. The fragments hybridising with omegal-DNA in the preliminary tests are electroeluted and purified via Elutip[®] columns (Schleicher & Schüll). These fragments are ligated with HindIII-linearised dephosphorylated pUC8 (Messing, J., Vieira, J., Gene 19, 269-276

(1982)) and *E. coli* JM101 (supE, thi, (lac-proAB), [F', traD36, pro AB, lac q Z M15] (e.g. P.L. Biochemicals) is transformed with the ligase reaction solution. The bacteria are spread on LB agar containing 50 µg/ml of ampicillin, 250 µg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BCIG, Sigma) and 250 µg/ml of isopropyl-β-D-thiogalacto-pyranoside (IPTG, Sigma). Blue colouration of the colonies formed indicates the absence of an insert in pUC8. The plasmid DNA's were isolated on a small scale from some white clones, then cut with HindIII and separated on 1% agarose gels. The DNA fragments were transferred to nitrocellulose filters and hybridised with ³²P-omegal-DNA as above. Starting from cos9 and cos10, a subclone was selected in each case and designated pRH22 or pRH57. From cosB, two DNA fragments which hybridise well with the omegal probe were subcloned and designated pRH51 or pRH52.

c) Sequence analysis

The DNA inserted in pUC8 is separated from the vector part by cutting with HindIII and subsequent gel electrophoresis. This DNA, about 10 µg, is ligated in 50 µl of reaction solution using T₄ DNA ligase, the volume is adjusted to 350 µl with nick translation buffer (Example 4d) and then decomposed using ultrasound, whilst cooling with ice (MSE 100 Watt ultrasonic disintegrator, maximum output at 20 kHz, five times 30 seconds). Then the ends of the fragments are repaired by adding 1/100 vol of 0.5 mM dATP, dGTP, dCTP and dTTP and 10 units of Klenow fragment of the DNA polymerase I for 2 hours at 14°C. The resulting DNA fragments having straight ends are separated according to their size on a 1% agarose gel. Fragments of sizes between 500 and 1000 bp were isolated and subcloned in

the dephosphorylated phage vector M13 mp8 cut with SmaI. The single strand DNA of recombinant phages is isolated and sequenced using the method developed by Sanger (Sanger, F. et al., Proc. Natl. Acad. Sci 74, 5463-5467 (1976)). The individual sequences are put together to form the total sequence by means of computers (Staden, R., Nucl. Acids Res. 10, 4731-4751 (1982)).

d) Sequence of the subclone pRH57 (IFN-omegal)

The sequence is shown in Figure 11. This fragment which is 1933 bp long contains the gene for interferon-omegal. The region coding for protein comprises the nucleotides 576 to 1163. The sequence is totally identical to that of the cDNA clone P9A2. The nucleotide portion 576 to 674 codes for a signal peptide 23 amino acids long. The TATA box is at a spacing characteristic of interferon Type I genes in front of the starting codon ATG (positions 476-482). The gene has a number of signal sequences for polyadenylation during transcription (ATTAAA at positions 1497-1502, or 1764-1796; AATAAA at positions 1729-1734 or 1798-1803), the first of which was used in the clone P9A2.

e) Sequence of the subclone pRHW22 (IFN-pseudo-omega2)

Figure 12 shows the sequence, 2132 bp long, of the HindIII fragment from the cosmid cos9 which hybridises with the omegal-DNA sample. An open reading frame is produced from nucleotide 905 to nucleotide 1366. The amino acid sequence derived therefrom is shown. The first 23 amino acids are similar to those of the signal peptide of a typical Type I interferon. The following 131 amino acids show a similarity to omegal interferon, up to amino acid 65, whilst tyrosine is notable as the first

amino acid of the mature protein.

Following amino acid position 66 is the sequence of a potential N-glycosylation site (Asn-Phe-Ser). From this point onwards the amino acid sequence is different from that of a Type I interferon. However, it can be demonstrated that similarity to the IFN-omegal can be established by suitable insertions and the resulting displacement of the protein reading frame (see Example 9). Thus, from the standpoint of Type I interferons, the isolated gene is a pseudogene: IFN-pseudo-omega2.

f) Sequence of the subclone pRH51 (IFN-pseudo-omega3)

The HindIII fragment originating from cosmid B and about 3500 bp long which hybridises with the omegal probe is partially sequenced (Figure 13). An open reading frame is obtained from nucleotide position 92 to 394. The first 23 amino acids display the features of a signal peptide. The subsequent sequence starts with tryptophan and shows similarity to the IFN-omegal up to amino acid 42. Thereafter, the derived sequence is different from IFN-omegal and ends after amino acid 78. The sequence can be altered by insertions so that it is then greatly homologous to IFN-omegal (Example 9). The gene is designated IFN-pseudo-omega3.

g) Sequence of the insert of pRH52 (IFN-pseudo-omega4)

The sequence of the HindIII fragment which is 3659 bp long, is isolated from cosmid B and which hybridises with omegal-DNA, is shown in Figure 14. An open reading frame the translation product of which is partially homologous to IFN-omegal is located between nucleotide positions 2951 and 3250. After a signal peptide 23 amino acids long, the further amino acid sequence begins with phenylalanine.

Homology to IFN-1 is interrupted after only the 16th amino acid, continues at the 22nd amino acid and ends at the 41st amino acid. Translation would be possible up to amino acid 77. Analogously to Example 8f) and 8g), good homology can be established with IFN-omegal by the introduction of insertions (Example 9). The pseudo gene isolated here is designated IFN-pseudo-omega4.

Example 9

Evaluation of the genes for 4 members of the IFN-omegal family

Figure 15 lists the genes for IFN-omegal to IFN-pseudo-omega4 one below the other together with the amino acid translation. To establish better concordance, gaps are inserted in the individual genes which are indicated by dots. No bases are omitted. The numbering of the bases includes the gaps. The amino acid translation of IFN-omegal is retained (e.g. at positions 352-355: "C.AC" codes for His). In the case of the pseudo genes, translation into an amino acid is given only where this is clearly possible. This list immediately shows that the 4 isolated genes are related to one another. Thus, for example, the potential N-glycosylation point (nucleotide positions 301 to 309) is obtained in all 4 genes.

Similarly, apart from the case of IFN-pseudo-omega4, at nucleotide positions 611 to 614, there is a triplet which represents a stop codon and which, in the case of IFN-omegal, terminates a mature protein with a length of 172 amino acids. Certainly, in this arrangement, there are premature stop codons in IFN-pseudo-omega2 (nucleotide positions 497 to 499) and in IFN-pseudo-omega4 (nucleotide positions 512 to 514).

The degree of relationship between the genes or the amino acid translations can be calculated from the arrangement shown in Figure 15. Figure 16 shows the DNA homologies between the members of the IFN-omega family. In the comparison in pairs, those positions where one of the two partners or both partners have a gap are not included in the counting. The comparison gives a homology of about 85% between IFN-omega1-DNA and the sequences of the pseudo genes. IFN-pseudo-omega2-DNA is about 82% homologous to the DNA's of IFN-pseudo-omega3 and IFN-pseudo-omega4. Figure 17 shows the results of comparisons of the signal sequences and Figure 18 shows the results of the comparisons of the "mature" proteins. The latter vary between 72 and 88%. However, this homology is substantially greater than that between IFN-omega1 and the IFN- α 's and IFN- β (Example 6). The fact that the individual members of the IFN-omega family are more remote from one another than the members of the IFN- α family can be explained by the fact that three of the four isolated IFN-omega-genes are pseudo genes and are clearly not subject to the same selection pressure as functional genes.

Example 10

Fermentation

Care of the strain:

A single colony of the strain HB101/pRHW12 on LB-agar (with 25 mg/ml of ampicillin) is super-inoculated with 25 mg/ml of ampicillin in trypton-soya broth (OXDID CM129) and shaken at 250 rpm at 37°C until an optical density (546 nm) of about 5 is achieved (logarithmic growth phase). Then 10 wt.-% of sterile glycerol are added, the culture is transferred into sterile ampoules in 1.5 ml batches and frozen at -70°C.

Primary culture:

The culture medium contains 15 g/l $\text{Na}_2\text{HPO}_4 \times 12\text{H}_2\text{O}$; 0.5 g/l NaCl; 1.0 g/l NH_4Cl ; 3.0 g/l KH_2PO_4 ; 0.25 g/l $\text{MgSO}_4 \times 7\text{H}_2\text{O}$; 0.011 g/l CaCl_2 ; 5 g/l caseine hydrolysate (Merck 2238); 6.6 g/l glucose-monohydrate; 0.1 g/l ampicillin; 20 mg/l cysteine and 1 ml/l thiamine-hydrochloride. 4 x 200 ml of this medium, each in a 1000 ml Erlenmeyer flask, are inoculated with 1 ml of a stock culture of HB101/pRHW14 and shaken for 16 to 18 hours at 37°C and at 250 rpm.

Main culture:

The medium for fermentation contains 10 g/l $(\text{NH}_4)_2\text{HPO}_4$; 4.6 g/l $\text{K}_2\text{HPO}_4 \times 3\text{H}_2\text{O}$; 0.5 g/l NaCl; 0.25 g/l $\text{MgSO}_4 \times 7\text{H}_2\text{O}$; 0.011 g/l CaCl_2 ; 11 g/l glucose-monohydrate; 21 g/l casein-hydrolysate (Merck 2238); 20 mg/l cysteine, 1 mg/l thiamine-hydrochloride and 20 mg/l 3- β -indolacrylic acid. 8 litres of sterile medium in a fermenter with a total volume of 14 litres (height: radius = 3:1) are inoculated with 800 ml of the primary culture.

Fermentation is carried out at 28°C, 1000 rpm. (Effigis turbine), at an aeration rate of 1 vvm (volume/volume/minute) and at a starting pH of 6.9. During fermentation the pH level falls and is then maintained constant at 6.0 using 3N NaOH. Once an optical density (546 nm) of 18 to 20 is reached (usually after 8.5 to 9.5 hours fermentation), the preparation is cooled to 20°C under otherwise identical conditions and then adjusted to a pH of 2.2 using 6N H_2SO_4 (without aeration) and stirred for 1 hour at 20°C and at 800 rpm (without aeration). The biomass activated in this way is then centrifuged at 30,000 rpm in a CEPA laboratory centrifuge Type GLE and frozen and stored at -20°C.

Example 11

Purification of interferon-omega-Gly

a) Partial purification

All the steps are carried out at 4°C.

140 g of biomass (E. coli HB101 transformed with the expression clone pRHW12) are resuspended in 1150 ml of 1% acetic acid (pre-cooled to 4°C) and stirred for 30 minutes. The suspension is adjusted to pH 10.0 using 5 M NaOH and stirred for a further 2 hours. After the pH has been adjusted to 7.5 using 5 M HCl, the mixture is stirred for a further 15 minutes and then centrifuged (4°C, 1 hour at 10,000 rpm, J21 centrifuge (Beckman), JA10 rotor).

The clear supernatant solution is poured onto a 150 ml CPG(controlled pore glass) column (CPG 10-350, mesh size 120/200) at a rate of 50 ml/h, washed with 1000 ml of 25 mM tris/1M NaCl, pH 7.5, and eluted with 25 mM tris/1M KCNS/50% ethyleneglycol, pH = 7.5 (50 ml/h).

The protein peak containing the interferon activity is collected, dialysed against 0.1 M Na-phosphate, pH = 6.0 and 10% polyethyleneglycol 40,000 overnight and the precipitate formed is removed by centrifuging (4°C, 1 hour, 10,000 rpm, J21 centrifuge, JA20 rotor) (see Table 1).

b) Further purification

The dialysed and concentrated CPG eluate is diluted with buffer A (0.1 M Na-phosphate pH = 6.25/25% 1,2-propyleneglycol) 1:5 and applied, by means of a "Superloop" injection apparatus (Pharmacia) at a rate of 0.5 ml/min, to the MonoS® 5/5 column (Pharmacia, cation exchanger) equilibrated with buffer A. Elution is carried out with 20 ml of a linear gradient from 0 to 1 M NaCl in buffer A, again at a flow rate of

0.5 ml/min. The throughflow and the 1 ml fractions were collected and tested for interferon activity by means of a CPE reduction test. The active fractions were collected.

Table 1

	Vólume (ml)	Biological U/ml ⁺	Test U total	Protein (mg/ml)	total (mg)	U/mg	Yield %
crude	1150	15000	$17,3 \times 10^6$	3,6	4140	4180	100
DL	2200	< 600	$< 1,3 \times 10^6$	0,74	1628	<600	< 5
Eluate	124,3	170000	$21,0 \times 10^6$	16,8	2088	10000	121
after dialysis	41	300000	$12,3 \times 10^6$	12,6	516,6	23800	71

* CPE reduction test: A549 cells, EMC virus

U: Units based on interferon- $\alpha 2$ (see Example 2 of EP-A-0.115.613: E. coli HB101 transformed with the expression clone pER 33) as standard

DL: throughflow

Example 12

Characterisation of HuIFN-omegal

A. Antiviral activity on human cells

B. Antiviral activity on monkey cells

C. Antiproliferative activity on human Brukitt's lymphoma cells (Daudi cell line)

D. Antiproliferative activity on human cervical carcinoma cells (HeLa cell line)

E. Acid stability

F. Serological characterisation

A. Antiviral activity on human cells

Cell line: Human lung carcinoma cell line A549
(ATCC CCL 185)
Virus: Murine encephalomyocarditis virus (EMCV)
Test system: Inhibition of cytopathic effect (all
titrations were carried out four times)

A partially purified preparation of HuIFN-omegal with a protein content of 9.4 mg/ml was titrated in a suitable dilution in the above-mentioned test system. The preparation showed an antiviral activity with a specific activity of 8300 IU/mg based on the reference standard Go-23-901-527.

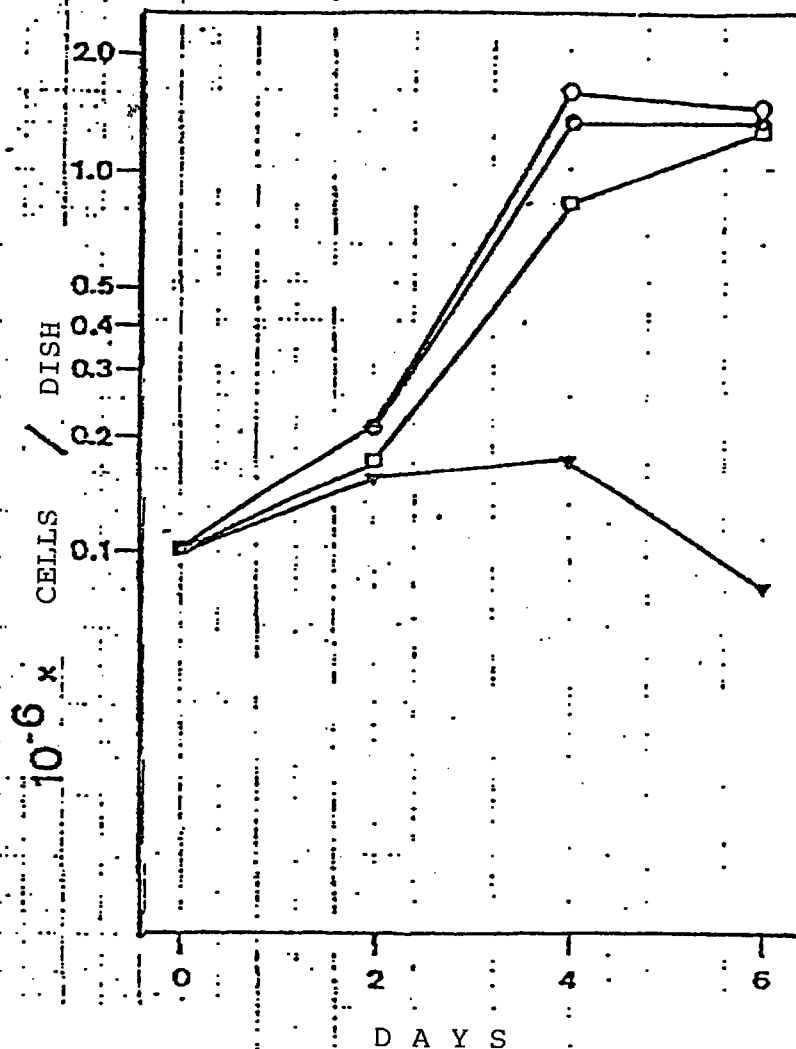
B. Antiviral activity on monkey cells

Cell line: G1-V3 monkey kidney cells (Christofinis
G.J., J. Med. Microbiol. 3, 251-258, 1970)
Virus: Vesicular Stomatitis Virus (VSV)
Test system: Plaque-Reduction Test (all titrations were
carried out four times)

The preparation described in Example 12A showed a specific antiviral activity of 580 U/mg in the test system mentioned.

C. Antiproliferative activity on human Burkitt's lymphoma cells (Daudi cell line)

The human Burkitt's lymphoma cell line Daudi was grown in the presence of various concentrations of HuIFN-omegal. After two, four and six days incubation at 37°C the cell density was determined; untreated cultures were used as controls. All the cultures were carried out three times in parallel. The Figure which follows shows that IFN-omega exhibits a marked inhibition of cell proliferation at a concentration of 100 antiviral units (IU, see Example 12A) per ml; at a concentration of 10 IU/ml a partial transient inhibition was observed (the following symbols are used in the Figure which follows: O control, o 1 IU/ml, □ 10 IU/ml, ▽ 100 IU/ml):



D. Antiproliferative activity on human cervical
carcinoma cells (cell line HeLa)

The human cervical carcinoma cell line HeLa was grown in the presence of the following proteins or protein mixtures:

HuIFN-omegal (see Example 12A) 100 IU/ml

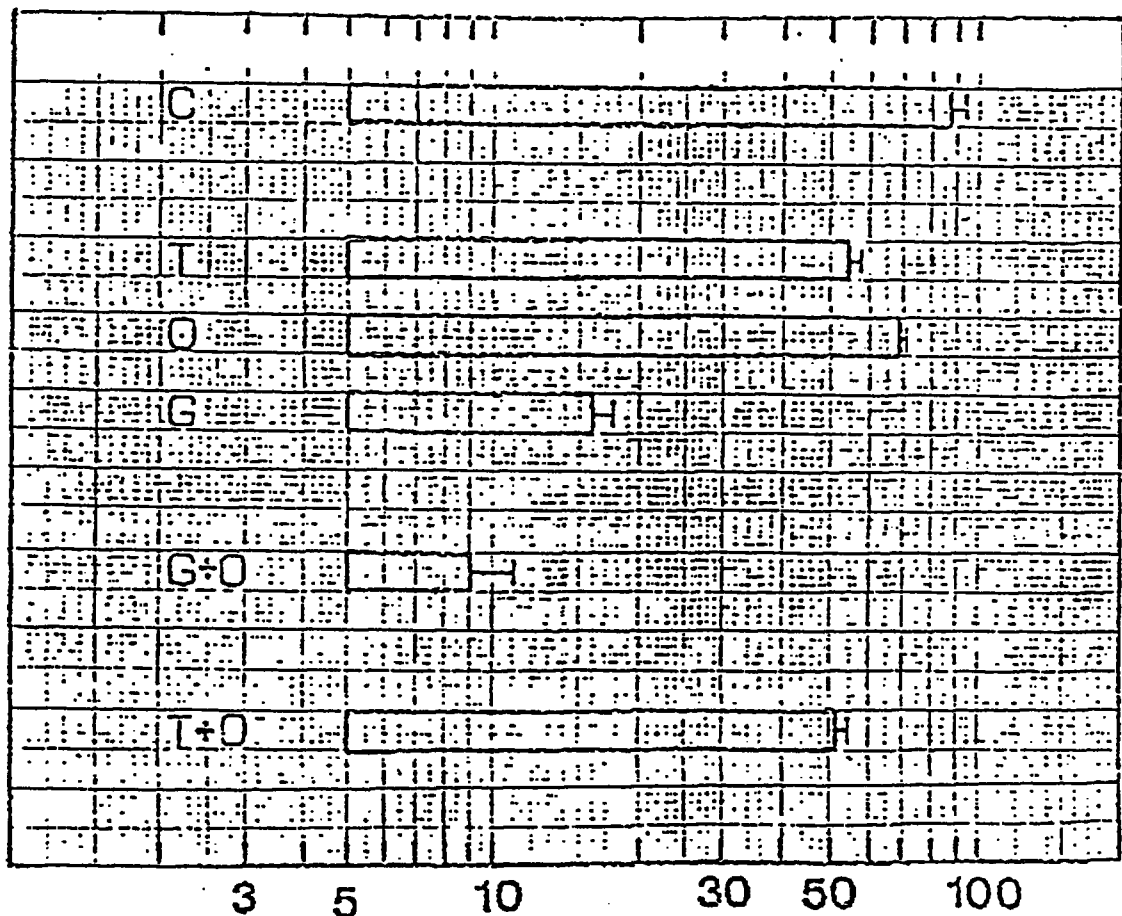
HuIFN-gamma (see Example 12A) 100 IU/ml

Human tumour necrosis factor (HuTNF) $\geq 98\%$ pure, made by Genentech Inc., San Francisco, USA (see Pennica D. et al., Nature 312, 724-729; 1984) 100 ng/ml

All the binary combinations of the said proteins
Concentrations as above

2 Cultures were set up in 3 cm plastics tissue culture dishes (50,000 cells per dish) and incubated for six days at 37°C; then the cell density was determined. Treatment of the cells with HuIFN-omegal or HuTNH-gamma had only a slight effect on cell growth, whereas HuIFN-gamma showed a significant cytostatic effect. Combinations of IFN-gamma with IFN-omegal showed synergistic cytostatic/cytotoxic effect.

The following Figure gives the results of the experiment:



$10^{-4} \times$ CELLS / DISH

The following symbols are used in the Figure:

C = untreated control, T = HuTNF, O = HuIFN-omegal,
G = HuIFN-gamma.

E. Acid stability

In order to investigate the acid stability of HuIFN-omegal a dilution of the preparation mentioned in Example 12A in cell culture medium (U/min.I 1640 with 10% foetal calves' serum) was adjusted to pH 2 using HCl, incubated at 4°C for 24 hours and then neutralised with NaOH. This sample was titrated in the antiviral test (see Example 12A); it showed 75% of the biological activity of a control incubated at neutral pH. IFN-

omegal can thus be regarded as acid-stable.

F. Serological characterisation

In order to determine the serological properties of HuIFN-omegal in comparison with HuIFN- α 2, samples (diluted to 100 IU/ml) were pre-incubated with equal volumes of solutions of monoclonal antibodies or polyclonal antisera for 90 minutes at 37°C; the antiviral activity of the samples was then determined. The following Table shows that HuIFN-omegal can only be neutralised by an antiserum against leukocyte interferon in relatively high concentrations but cannot be neutralised by antibodies directed against HuIFN- α 2 or HuIFN-beta. HuIFN-omegal is therefore not serologically related either to HuIFN- α 2 or to HuIFNbeta:

Antiserum monoclonal antibodies	Dilution µg/ml	Neutralisation of	
		HuIFN-α2	HuIFN-ωegal
EBI-1 ¹⁾	1	+	-
	10	+++	-
	100	+++	-
	1000	-	0
EBI-3 ¹⁾	1	+++	-
	10	+++	-
	100	+++	-
	1000	+++	0
L3B7 ²⁾	100	+++	0
	1000	+++	0
Sheep anti- leukocyte-IFN ³⁾	1:50,000	+++	-
	1: 5,000	+++	0
	1: 500	+++	+
	1: 50	-	+++
Rabbit anti- HuIFN-α2	1: 1,000	+++	-
	1: 100	+++	0
	1: 10	-	0
Sheep anti- HuIFN-β ⁴⁾	1: 50	-	0

¹⁾ = see EP-A-0.119.476

²⁾ = A. Berthold et al. in *Arzneimittelforschung* 35,
364-369 (1985)

³⁾ = Research Reference Reagent Catalog No.
G-026-502-568

⁴⁾ = Research Reference Reagent Catalog No.
G-028-501-568
Research Resources Branch, National Institute of
Allergy and Infectious Diseases, Bethesda,
Maryland, USA.

Symbols: - not tested, 0 no neutralisation, + partial neutralisation, +++ complete neutralisation

56484J.18



Patent Claims

1. Recombinant DNA molecule containing a coding sequence for new human interferon proteins of type I, containing 168-174 amino acids, for interferon-pseudo-omega2 (see Figure 12), interferon-pseudo-omega3 (see Figure 13) or interferon-pseudo-omega4 (see Figure 14), characterised in that the coding sequence is hybridised under stringent conditions which enable a homology greater than 85% to be recognised, with the inserts in the Pst-1 cutting site a) of the plasmid E76E9 in E. coli HB 101, filed at the DSM under the number DSM 3003 or b) of the plasmid P9A2 in E. coli HB 101, filed at the DSM under the number DSM 3004.
2. DNA molecule as claimed in claim 1, characterised in that the stringent conditions only enable a homology of more than 90% to be detected.
3. DNA molecule as claimed in claims 1 and 2, characterised in that the coding sequence is present as an insert in the Pst-1 cutting site a) of the plasmid E76E9 in E. coli HB 101, filed at the DSM under the number DSM 3003 or b) of the plasmid P9A2 in E. coli HB 101, filed at the DSM under the number DSM 3004, or a degenerate variation of this insert.
4. DNA molecule as claimed in claims 1 to 3, characterised in that the vehicle is a plasmid which is replicatable in procaryotes or eucaryotes.
5. DNA molecule according to claim 4, characterised in that it is an expression vehicle which is replicatable in microorganisms or in mammalian cells.
6. DNA molecule as claimed in claim 5, containing the sequence



TGT GAT CTG CCT CAG AAC CAT GGC CTA CTT AGC AGG AAC ACC TTG 45
GTG CTT CTG CAC CAA ATG AGG AGA ATC TCC CCT TTC TTG TGT CTC 90
AAG GAC AGA AGA GAC TTC AGG TTC CCC CAG GAG ATG GTA AAA GGG 135
AGC CAG TTG CAG AAG GCC CAT GTC ATG TCT GTC CTC CAT GAG ATG 180
CTG CAG CAG ATC TTC AGC CTC TTC CAC ACA GAG CGC TCC TCT GCT 225
GCC TGG AAC ATG ACC CTC CTA GAC CAA CTC CAC ACT GGA CTT CAT 270
CAG CAA CTG CAA CAC CTG GAG ACC TGC TTG CTG CAG GTA GTG GGA 315
GAA GAA GAA TCT GCT GGG GCA ATT AGC AGC CCT GCA CTG ACC TTG 360
AGG AGG TAC TTC CAG GGA ATC CGT GTC TAC CTG AAA GAG AAG AAA 405
TAC AGC GAC TGT GCC TGG GAA GTT GTC AGA ATG GAA ATC ATG AAA 450
TCC TTG TTC TTA TCA ACA AAC ATG CAA GAA AGA CTG AGA AGT AAA 495
GAT AGA GAC CTG GGC TCA TCT 516

7. DNA molecule as claimed in claim 5, characterised in that the DNA molecule as claimed in claim 6 contains the nucleotide A instead of the nucleotide G in position 332.

8. DNA molecule as claimed in claim 5, characterised in that it is a) the plasmid E76E9 in E. coli HB 101 filed at the DSM under the number DSM 3003 or b) the plasmid P9A2 in E. coli HB 101, filed at the DSM under the number DSM 3004.

9. DNA molecule as claimed in claims 6 and 7, characterised in that it additionally contains the DNA sequence of formula

ATG GCC CTC CTG TTC CCT CTA CTG
GCA GCC CTA GTG ATG ACC AGC TAT AGC CCT GTT GGA TCT CTG GGC
coding for the leader peptide.



10. Interferon-omega1 gene of formula

GATCTGGTAAACCTGAA 17
GCAAATATAGAAACCTATAGGGCCTGACTTCCTACATAAAGTAAGGAGGGTAAAAATGG 76
AGGCTAGAATAAGGGTTAAAATTTTGCTTCTAGAACAGAGAAAATGATTTTTTTCATAT 135
ATATATGAATATATATTATATATACACATATATACATATATTCACTATAGTGTGTATAC 194
ATAAATATATAATATATATATTGTTAGTGTAGTGTGTGTCTGATTATTTACATGCATAT 253
AGTATATACACTTATGACTTTAGTACCCAGACGTTTTTCATTTGATTAAGCATTCATTT 312
GTATTGACACAGCTGAAGTTTACTGGAGTTTAGCTGAAGTCTAATGCAAAATTAATAGA 371
TTGTTGTCATCCTCTTAAGGTCATAGGGAGAACACACAAATGAAAACAGTAAAAGAAAC 430
TGAAAGTACAGAGAAATGTTTCAGAAAATGAAAACCATGTGTTTCCTATTAAAAGCCATG 489
CATACAAGCAATGTCTTCAGAAAACCTAGGGTCCAAGGTTAAGCCATATCCCAGCTCAG 548
TAAAGCCAGGAGCATCCTCATTTCCCA ATG GC₃ CTC CTG TTC CCT CTA CTG 599
GCA GCC CTA GTG ATG ACC AGC TAT AGC CCT GTT GGA TCT CTG GGC 644
TGT GAT CTG CCT CAG AAC CAT GGC CTA CTT AGC AGG AAC ACC TTG 689
GTG CTT CTG CAC CAA ATG AGG AGA ATC TCC CCT TTC TTG TGT CTC 734
AAG GAC AGA AGA GAC TTC AGG TTC CCC CAG GAG ATG GTA AAA GGG 779
AGC CAG TTG CAG AAG GCC CAT GTC ATG TCT GTC CTC CAT GAG ATG 824
CTG CAG CAG ATC TTC AGC CTC TTC CAC ACA GAG CGC TCC TCT GCT 869
GCC TGG AAC ATG ACC CTC CTA GAC CAA CTC CAC ACT GGA CTT CAT 914
CAG CAA CTG CAA CAC CTG GAG ACC TGC TTG CTG CAG GTA GTG GGA 959
GAA GGA GAA TCT GCT GGG GCA ATT AGC AGC CCT GCA CTG ACC TTG 1004
AGG AGG TAC TTC CAG GGA ATC CGT GTC TAC CTG AAA GAG AAG AAA 1045
TAC AGC GAC TGT GCC TGG GAA GTT GTC AGA ATG GAA ATC ATG AAA 1094
TCC TTG TTC TTA TCA ACA AAC ATG CAA GAA AGA CTG AGA AGT AAA 1139
GAT AGA GAC CTG GGC TCA TCT TGA AATGATTCTCATTGATTAATTTGCCAT 1190
ATAACACTTGCACATGTGACTCTGGTCAATTCAAAGACTCTTATTTTCGGCTTTAATCA 1249
CAGAATTGACTGAATTAGTTCTGCAAATACTTTGTCGGTATATTAAGCCAGTATATGTT 1308
AAAAAGACTTAGGTTTCAGGGGCATCAGTCCCTAAGATGTTATTTATTTTTACTCATTTA 1367
TTTATTCTTACATTTTATCATATTTATACTATTTATATTCTTATATAACAAATGTTTGC 1426
CTTTACATTGTATTAAGATAACAAAACATGTTTCAGCTTTCCATTGGTTAAATATTGTA 1485
TTTTGTTATTTATTAAATTATTTTCAAACAAAACCTCTTGAAGTTATTTATTCGAAAAC 1544
CAAAATCCAAACACTAGTTTTCTGAACCAAATCAAGGAATGGACGGTAATATACACTTA 1603
CCTATTCATTTCATTCCATTTACATAATATGTATAAAGTGAGTATCAAAGTGGCATATTT 1662
TGGAATTGATGTCAAGCAATGCAGGTGTACTCATTGCATGACTGTATCAAATATCTCA 1721
TGTAACCAATAAATATATACACTTACTATGTATCCCACAAAAATTAAAAAGTTATTTTA 1780
AAAAAGAAATACAGGTGAATAAACACAGTTTCTTTCCGTGTTGAAGAGCTTTCATTCTT 1839
ACAGGAAAAGAAACAGTAAAGATGTACCAATTTTCGCTTATATGAAACACTACAAAGATA 1898
AGTAAAAGAAAATGATGTTCTCATACTAGAAGCTT 1933



11. Interferon-pseudo-omega2-gene of formula

AAGCTTGAGCCCCCAGGGAAGCATAACCACATGAACCTGAATGAATATATT 51
CTAGAAGGAGGGAAGCACCAGAGAAGTTCTTTCACTAATAACCATCAACGTCTTCTGTG 110
AATCAAATATCAAACAAAGATAGTCCTAAAAAGTTTAATTTCCAGAGATAGGTAATTC 169
CTAACTGAATACAGAAACCCATAGGGCCCAGGGATCCTGATTTCTATGCAAAATGGAG 228
GGTAAAACTGGAGGCTAGGATCTGGGCTAAAAGTATATACTTCTAACAGTAGCACAAAG 287
ATGTTTCTCATCTGATTGATCAATATTCATTTGGATTGATATATCTTAAGTTTACTGGG 346
AATATTGAACATCCATTGCAAAAATCAAGAGTGTAGAGTGATGACCTCCTTTTAGGTCA 405
TATAGAACAAGGTTTTTCAACCCCCATCCATGGACCGGGGTACTGGTCCTGGCCTGGTA 464
GGAACAGGGCCGCACAGCAGGAGGCAAGCAGGCCAACCAACAAGCATTAACGCCTGAGC 523
TCTGCCTCCTGTCAGATCAGCAGTGGCATTGGATTCTCAAAGAGCAGGAACCCTATTG 582
TGAAGTGCAGATGCGAAGGATCTAGGTTGTGGTCTCCTAATGAGAATCTAATGCCTCTG 641
AAAGCATTCCCTCCCTGACCCCATTTTTTCGTGGAAAAATTATCTTCCACCAAACCTGGTG 700
GCCAAAAGGTTGTGGATGCTGATATAGAAGACATGTAAATGAAAACAATAAATGGAATT 759
AAAAATTTAGAGAAATGCTCAGAAAAATGAAAACCTATTTGTGCTCCATTAAAGCCATGC 818
ATAGATAGAATGTCTTCATAGAACCTAGGATCCAAGGTTCTATGAAGACCTCAGCTCAA 877
CCAGGCCAAAAGCATCCTGATTTCTCA ATG GCC CTC CTC TTC CCT CTA CTG 928
GCA GCC CTA GAG GTG TGC AGC TGT GGC TCT TCT GGA TCT CTA GGA 973
TAT AAC CTG CCT CAG AAC CAT GGC CTG CTA GGC AGG AAC ACC TTG 1018
GTG CTT TTG GGC CAA ATG AGG AGA ATC TCT CCC TTC TTG TGT CTA 1063
AAG GAC AGA AGT GAC TTC AGA TTC CCC CAG GAG AAG GTG GAA GTC 1108
AGC CAG TTG CAG AAG GCC CAG GCT ATG TCT TTC CTC TAT GAT GTG 1153
TTA CAG CAG GTC TTC AAC TTC TCA CAC AAA GCG CTC CTC TGC TGC 1198
ATG GAA CAT GAC CTT CCT GGA CCA ACT CCA CAC TTT ACG TCA TCA 1243
GCA GCT GGA ACA CCT GGA GAC CTG CTT GGT GCA GGA GAT GGG AGA 1288
AGG AGA AGC TGG GGG CAG TGG GTG ATT GAG GGC TCT ACA CTG GCC 1333
TTG AGG AGG TAT TTC CAG GAA TCC ATC TCT ACC TGA AAGAGAAGAAA 1380
TAAAGATTGTGCCTGGGAAGTTGTCAGAGTGGAAATCATGAGATCCTTTTCATCCACAA 1439
GTTTGCAAGAAAGATTGAGAAGTAAGGATGAAGACCTGGGCTCATCATGAAATGATTCT 1498
CATTGACTAATCTGCCATATCACACTTGTACATGTGACTTTGGATATTCAAAAAGCTCA 1557
TTTCTGTTTCATCAGAAATTATTGAATTAGTTTTAGCAAATACTTTATTAATAGCATAA 1619
AGCAAGTTTATGTCAAAAACATTCAGCTCCTGGGGCATCCGTAATCAGAGATAACTGC 1675
CCTGATGCTGTTTATTTATCTTCCCTTCTTTTTTTTCATGCCTTGATTTTATGATATTTA 1734
TATATTTTATATTTTCATCTTCACATCGTATTAAAATTTATAAAACATTCACTTTTTTCA 1793



TATTAAGTTTGCATTTTGT TTTTATTA AATTCATATCAAAGAAA CTCTGTAAATGTTTC 1852
TATTCTAAAAACAATGTCTACTTTCTCTTTTGTAAACCAAATTGAAAATATGGTAAAA 1911
TGTATTAAC TCATTCATTTCA TTCCTATTATATGTATAAATTGAGTAAATGGCAA ACTG 1970
TGGGGT TTTCTTAAAGAAATACAGGTGAATAAAGCAAACACAGTTTCTCTCAGTCTAAG 2029
AGGGAAAGAGACGTAAAAACAGGACAAATATTTATATTATTCAATTATGTTAAATGCT 2088
ACAAAGAGAAGTAAAGAAAAGTGATGTTCTCACATCAGAAGCTT 2132

12. Interferon-pseudo-omega3-gene of formula

CCATG 5

CATAGCAGGAATGCCTTCAGAGAACCTGAAGTCCAAGGTT CATCCAGACCCCAGCTCAG 64
CTAGGCCAGCAGCACCCCTCGTTTCCCA ATG GTC CTC CTG CTT CCT CTA CTC 115
GTG GCC CTG CCG CTT TGC CAC TGT GGC CCT GTT GGA TCT CTG AGC 160
TGG GAC CTG CCT CAG AAC CAT GGC CTA CTT AGC AGG AAC ACC TTG 205
GCA CTT CTG GGC CAA ATG TGC AGA ATC TCC ACT TTC TTG TGT CTC 250
AAG GAC AGA AGA GAC TTC AGG TTC CCC CTG GAG ATG TGG ATG GCA 295
GTC AGT TGC AGA AGG CCC CGG CCC TGT CTG TCC TCC ATG AGA TGC 340
TTC AGC AGA TCT TCA GCC TCT TCC CCA CAG AGT GCT CCT CTG CTG 385
CCT GGA ACA TGA CCCTCCTGGACCAACTCCACACTGGACTTCATCTGCAGCTGGA 440
ATGCCTGGATGCTTGCTTAGGGCAGACAAAAAGAGAGGAAGAATCTGTGGGGGTGATTG 499
GGGCCCTACACTGGCCTTGAGGACGTACTTTCAGGGAATGCATGGGAATCCAGGGAATC 558
TACCTGGAGGAGAAGAAATACAGTGACTGTGCTTGGGAGGTTGTCAGAGTGGAATCGTG 617
AAATCCTTCTCTTCATCATCAACAACTTGCAAGAAGGACTGAGAAGTAAGGATGAAGA 676
CCTGGGTTCATCCTGAAATTATTCTCATTGATTAATCTGCCATATCACACTTGCACATG 735
TGTCTTTGGTCATTTCAATAGGTTCTTATTTCTGCAG 772

13. Interferon-pseudo-omega4 gene of formula

AAGCTTTGGGCATGACCTAGTAGGTGACTCTT 32

AGTTGGAGTGGTCAGTTGTAAGGCTCTTGCTCAGTCACGTGGCTCCCCTGTATTTCCCC 91
ACGTTTGCAGCCGTGCTCCTTCTCAATGCTATGAAAGTGTGGGCTCCTCTCCCACTGGA 150
GTGCTGACTGTAGCTTGTATCTTGGCACTCCCAGGCTGTACATAACAGCTCTGAGGTGA 209
TCTCAGGGTTAATGTTTTCTCCCCGACTTGGAGGCCATTGAAGGAAGGGACCTTAGTAG 268
TAGTTGTAAC TGAGGGTCTTTTGCTTGTCTCCTGGGGGCTCCACCCCAGAGATGCAGGT 327



GAGCAATCACTCAGTGCATTGAGCTTGGGATGGGGGTGTCTGTGCTGTGGGCCCAAGAC 386
AGGGGTTCCTGCCTGGTGATGTGAGGGGTGGGTGGTTGACCAATGGCAGACAGACTGG 445
CCTCCTCTCTTGGGTTGACAGCAGCTTGTGAGGTATGCATAAGGCACCTTGGGGTCTT 504
GCTCCTTCATTAGTTCCAAGGTAGCTGGGGTAGTACCACTGCAGAGGCAGTGTTAGACA 563
GGCTTTCTGTTACCCCTGGGGTCTCCACCTCCTAGAAATGTGAAGTCATGTTAATGGGA 622
GTGTTTAGCCAGAGGAGTGGGGTGGCTGCATGCTGGTGTAAGTATGGGACTTCACTTCT 681
TGGGAAACAGGGAGGTGGAATCTTACCAGCAGGAGACTGTTCTCCTCACGATGTGTAAC 740
CTGCAGTGTGCTGGAAGTTTAGGTGACTGTGGCATGATGTTAGCTTGTAACAAAGAGC 799
TTCAGACTCTTTGTCTCTTCCCCAGACCCAAGGCAGCAAGGATAAAAGCTGCTGCTGTG 858
GCAGTGGCAGAGGTAGGATGGTTGTGGGAGCCTCTCCCCAGGGAACTCTAGTCAACTA 917
CCAGTGGATATGCTCAGCCATGGGTAGGGTGACTGTTCTGCAGTCATGGGCAGGGGGCC 976
TGCTCCTGAAGAATAGGGACAGGGATTCTCAGGGAAGAGGGGCTGGA CTCTCTCATA 1035
TAGTGGCGATGATGTGCTGGAGGTGCCAGTGTAGTGAATAGGCCTTTGTTCTTCCCCA 1094
GCCAGAGGGCTGCTGGGGCTGTCCCACTGCAACGGTCATGGTGGATGGGTTATGGGTTG 1153
ACTGTGGGATTTCTTTCTTGGAGAAATGCTGGACTGCCTGATTGAGGAGACGAGGCAAG 1212
GGAAGAATGCCTGTGCTGGAGTCTCTGGTCAGGTGGCTCTGCCACAGAGGAGAGGTGAC 1271
CACCAGGAAGTGCCTGGAGAACAGTGTAGCCACTCTTCTGTGAGGCAGTTGCTCTGTTT 1330
TGGGGATCTGGAGCAGCCGCTATTCTTACAGATTCCCAGAGCCTGGAGACAGCAAGGG 1389
CAAGAGCTGTGAGAAAGCAAAGATAGCAACCCACCCTTCTCACTGGGAGCTCTGTTCCA 1448
GGGAGATGCAGAGCTGCCATTGCTCAATAGCCCCAGCTGGTAGCTGCAGACCCAGGCCT 1507
GGCAGACCCACCCAGTGAGCAGATAGGGGATTAGGGACCCACATAACACACAGTCTGGC 1566
CACTTTTCCATAGGGCTGCTGAATATGCTGGGGGTCCAATCCAGACCATAGTCACCTCA 1625
CATTTTTCAGTACCTGAAGATATCAACAGTGAAGGCTATGAAACAGTGAAGATGGGGAC 1684
CTGCCCCCTGCCTCTGGACCTCTGTTCCAGAGAGGTACAACCTGTTGCCTCCGACATACA 1743
TGCAGGAGGTGGCTGGAGACCCGGTGGATATCCCTCCCACTGAGGAGAAGCAGCATCAG 1802
GGAATCAGGTGAAGAAACAGTCTGGCCACTTTTTGGTAGAGCAGCTGTGCTTGCTGGGG 1861
GTCTGCTACCACCCCCAGCAAAAAGAATGGCATTGCAAGAATGGCTAAGGCTGCTAAA 1920
CAGCAACAATGGCAACCTACCATTTCCCTTTGGAGCGCCATCCCAGGGATATTGAAACT 1979
GCTGTCCACTAGAAAACAGTGGTGGAGGTGACTGGAGACCCAGTGGAGAGTTTCACCT 2038
GGTGAAAAGAAACAGGATTTGGGATCGACATGAATAACCAATCTGACTGCTTCCCCGTA 2097
GAGCTGCTGGACTGTGCTGGGTGGCTGCTCCAGTCCCTAGCTGCCTTGGACTCCCGAGA 2156
ACCCAAAGGCTCCAATAGCTAAGATTGTGAAAGAGCAAAGATGGCAGCCCACCCCTGC 2215
CACAGGGAGCTCCATGTCAGGGAGGTATGAGGCTGCTACCAGTGTCTGGCTGGATTCCC 2274
AAGTCGAGTGGGTCTTACCCTGAGACAGGCCATGGAAGGTGGGCCTGTCAATTGTCAGTG 2333
CCCAGCGCCCTGGATGAAACCCCTTTCTAGGGGTATGTATAGGGGTCTAGCGTCCTGC 2392
TTGGCTGGAGTTATAGCTTCTTTTGTGGGGAGGCCTGGGTATCTAAGGCTCCAGGGTAC 2451



CCATGCATGCGAGAGCGGCTGCTCTGCTGAACCCTACGTAGCCCTGCATGTCAGACTAA 2510
ATGCCCTGGTAGAGTGGGTTCCTAGGAGATCTCCTGACCTGAGGATTGCAAAGATCTG 2569
TGGGAGAAGCGTGGGTCCCCAGGGCTGCTCACTTACTCACCCTTCCCTGGGCAGGAGA 2628
GGCTCCCCTGGCTCTGTGTCATCCTGGGGGGGCAGTTGTCCTGCCTTACTTTGCTTTAT 2687
TCTCCATGGGTCAAGTTGTTTTCTTGAGTCTCAATGTGTGCACCTGGTTTTTTTCAGTTG 2746
AAGGTGCTGTATTTACTTGCCCCCTCCATTTCTCTCCATGAGAGTGGCACACACTAGCA 2805
GGTTCCAGTCGGCCATCTTGCAACCCCTGAAAATATTTGTTTCCAGCTATAAGCCATT 2864
GAGAGAACCTGGAGTGGCATAAAAAGAATGCCTCGGGGTTCATCCCGACCCCAGCTCAG 2923
CTAGGCCAGCAGCACCCTCGTTTCCCA ATG GTC CTA CTG CTT GTT CTA CTG 2974
GTG GCC CTG CTG CTT TGC CAA TGT GGC CCT GTT GGA TCT CTG GGC 3019
TTT GAC CTG CCT CAG AAC CGT GGC CTA CTT AGC AGG AAC ACC TTG 3064
GCA TTC TGG GCC AAA TGC AGA ATC TCC ACT TTC TTG TGT CTC AAG 3109
GAC AGA AGA GAC TTC AGG TTC CCC CTG GAG ATG TGG ATG GCA GTC 3154
ATT TGC AGA AGG CCC AGG CTG TGT CTG TCC TCC ATG AGA TGC TTC 3199
AGC AGA TCT TCA GCC TCT TCC CCA CAG AGC GCT CCT CTG CTG CCT 3244
GGA ACA TGA CCCTCCTGGACCAGCTCCACACTGGATTTTCATCAGCAGCTCGAATAG 3300
CCTGGAGTCTTGCTTAGGGCAGGCAACAGGAGAGGAAGAATCTGTGGGGGTGATTGGGA 3359
CCCTACACTGGCCTTGAGGAGGTA CTTCAGGGAATCCATGGGAATCCAGAGAATCTAC 3418
CTGAAAGAGAAGAAATACAGTGACTGTGCTTAGGAGGTTGTCAGAATGGAATCATGAAA 3477
TCCTTCTCTTCATCAACAGACTTGCAAGGACTGAGAAGTAAGGATGAAGACCTGGGGTC 3536
TGCTTTACTCTTTCTTATTTTCTTCTCCTTACTATGTGTTTATTTCTTCTTTTTC 3595
TAGTTCCTTA ACTTGTAAGTAGTTCACTTGGTTTGAGGTCTTTCTTCTTTTAAATATA 3654
AGCTT 3659

14. Transformed host organism which contains the genetic information as claimed in claims 1 to 10.
15. Host organism as claimed in claim 14, characterised in that the genetic sequence is contained in a vehicle which is replicatable in the host organism.
16. Host organism as claimed in claim 14, characterised in that it is a mammalian cell line or E. coli.
17. Host organism as claimed in claim 14, characterised in that it is E. coli with the DSM number 3003 or E. coli with the DSM number 3004.
18. Host organism as claimed in claim 17, characterised in that the vehicle additionally contains the replicon and control sequences required for expression.



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19. Host organism as claimed in claim 18, characterised in that the vehicle contains the replicon and control sequences of the plasmid pER103 with DSM no. 2773 (see Fig. 6) required for expression.

20. Host organism as claimed in claim 20, transformed with an expression plasmid as claimed in claims 21, 22 or 24.

21. Expression plasmid for interferon-omega which is a derivative of plasmid pBR 322, characterised in that instead of the shorter EcoRI/BamHI fragment inherent in the plasmid, the plasmid pBR322 contains the DNA sequence of formula

EcoRI Sau3A

qaattcacgctGATCGCTAAAACATTGTGCAAAAAGAGGGTTGACTTTGCCTTCGCGA 59

↓ mRNA-Start

Met

ACCAGTTAACTAGTACACAAGTTCACGGCAACGGTAAGGAGGTTTAAGCTTAAAG ATG 116

RBS

HindIII

Cys Asp

TGT GAT C — interferon-omega gene —>

Sau3A

22. Expression plasmid pRHW12 as claimed in claim 21, characterised in that the interferon-omega-gene contains the DNA sequence of formula



TGT GAT CTG CCT CAG AAC CAT GGC CTA CTT AGC AGG AAC ACC TTG 28
NcoI
GTG CTT CTG CAC CAA ATG AGG AGA ATC TCC CCT TTC TTG TGT CTC 73
AAG GAC AGA AGA GAC TTC AGG TTC CCC CAG GAG ATG GTA AAA GGG 118
AGC CAG TTG CAG AAG GCC CAT GTC ATG TCT GTC CTC CAT GAG ATG 163
CTG CAG CAG ATC TTC AGC CTC TTC CAC ACA GAG CGC TCC TCT GCT 208
GCC TGG AAC ATG ACC CTC CTA GAC CAA CTC CAC ACT GGA CTT CAT 253
CAG CAA CTG CAA CAC CTG GAG ACC TGC TTG CTG CAG GTA GTG GGA 298
GAA GGA GAA TCT GCT GAG GCA ATT AGC AGC CCT GCA CTG ACC TTG 343
AGG AGG TAC TTC CAG GGA ATC CGT GTC TAC CTG AAA GAG AAG AAA 388
TAC AGC GAC TGT GCC TGG GAA GTT GTC AGA ATG GAA ATC ATG AAA 433
TCC TTG TTC TTA TCA ACA AAC ATG CAA GAA AGA CTG AGA AGT AAA 478
GAT AGA GAC CTG GGC TCA TCT TGAAATGATTCTCATTTGATTAATTTGCCATA 530
TAACACTTGCACATGTGACTCTGGTCAATTCAAAAGACTCTTATTTTCGGCTTTAATCAC 589
AGAATTGACTGAATTAGTTCTGCAAATACCTTTGTCTGGTATATTAAGCCAGTATATGTTA 648
AAAAGACTTAGGTTACAGGGGCATCAGTCCCTAAGATGTTATTTATTTTACTCATTTAT 707
TTATTCTTACATTTTATCATATTTATACTATTTATATTCTTATATAACAAATGTTTGCC 766
TTTACATTGTATTAAGATAACAAAACATGTTCAGct 802
AluI

23. Expression plasmid pRHW11 as claimed in claim 21,
characterised in that the interferon-omega-gene contains
the DNA sequence of formula

TGT GAT CTG CCT CAG AAC CAT GGC CTA CTT AGC AGG AAC ACC TTG 28
NcoI
GTG CTT CTG CAC CAA ATG AGG AGA ATC TCC CCT TTC TTG TGT CTC 73
AAG GAC AGA AGA GAC TTC AGG TTC CCC CAG GAG ATG GTA AAA GGG 118
AGC CAG TTG CAG AAG GCC CAT GTC ATG TCT GTC CTC CAT GAG ATG 163
CTG CAG CAG ATC TTC AGC CTC TTC CAC ACA GAG CGC TCC TCT GCT 208
GCC TGG AAC ATG ACC CTC CTA GAC CAA CTC CAC ACT GGA CTT CAT 253
CAG CAA CTG CAA CAC CTG GAG ACC TGC TTG CTG CAG GTA GTG GGA 298



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GAA GGA GAA TCT GCT GAG GCA ATT AGC AGC CCT GCA CTG ACC TTG 343
AGG AGG TAC TTC CAG GGA ATC CGT GTC TAC CTG AAA GAG AAG AAA 388
TAC AGC GAC TGT GCC TGG GAA GTT GTC AGA ATG GAA ATC ATG AAA 433
TCC TTG TTC TTA TCA ACA AAC ATG CAA GAA AGA CTG AGA AGT AAA 478
GAT AGA GAC CTG GGC TCA TCT TGAAATGATTCTCATTGATTAATTTGCCATA 530
TAACACTTGACATGTGACTCTGGTCAATTCAAAAGACTCTTATTTCTGGCTTTAATCAC 589
AGAATTGACTGAATTAGTTCTGCAAATACTTTGTCTGGTATATTAAGCCAGTATATGTTA 648
AAAAGACTTAGGTTAGGGGCATCAGTCCCTAAGATGTTATTTATTTTACTCATTAT 707
TTATTCTTACATTTTATCATATTTATACTATTTATATTCTTATATAACAAATGTTTGCC 766
TTTACATTGTATTAAGATAACAAAACATGTTCAGct 802

AluI

24. Plasmid PRHW10 which is a derivative of plasmid pBR 322, characterised in that the DNA fragment of formula

HindIII	Sau3A	NcoI	
a <u>AGCTT</u> AAG	ATGTGT <u>GATC</u>	TGCCTCAGAA	<u>CCATGGCCTA</u> CTTAGCAGGA 50
ACACCTTGGT	GCTTCTGCAC	CAAATGAGGA	GAATCTCCCC TTTCTTGTGT 100
CTCAAGGACA	GAAGAGACTT	CAGGTTCCCC	CAGGAGATGG TAAAAGGGAG 150
CCAGTTGCAG	AAGGCCCATG	TCATGTCTGT	CCTCCATGAG ATGCTGCAGC 200
AGATCACACA TCTTTA <u>agct</u> t			
Sau3A	HindIII		

is inserted in the plasmid pER103, DSM no. 2773 (see Fig. 6), in the HindIII site.

25. E. coli HB 101 transformed with a plasmid as claimed in claim 21, 22, 23 or 24.

26. New human type I interferon proteins, characterised in that they are coded by a DNA molecule as claimed in one of claims 1 to 10.

27. New human interferons as claimed in claim 26, characterised in that the mature interferons have a



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divergence of 30 to 50% compared with human α -interferons.

28. New human interferons of type I as claimed in claim 26, characterised in that

a) the mature interferons have a divergence of 30 to 50% compared with human α -interferons and a divergence of approximately 70% compared with β -interferon and

b) they are 168 to 174 amino acids long, and the N-glycosylated derivatives thereof.

29. Interferons as claimed in claim 28, characterised in that they have a divergence of from 40 to 48% compared with human α -interferons, and the N-glycosylated derivatives thereof.

30. Interferons as claimed in claims 26 to 29, characterised in that they contain a leader peptide.

31. Interferons as claimed in claims 26 to 29, characterised in that they contain 172 amino acids.

32. Interferon as claimed in claim 31, characterised in that it contains the amino acid sequence

	5		10		15									
Cys	Asp	Leu	Pro	Gln	Asn	His	Gly	Leu	Leu	Ser	Arg	Asn	Thr	Leu
	20								25					30
Val	Leu	Leu	His	Gln	Met	Arg	Arg	Ile	Ser	Pro	Phe	Leu	Cys	Leu
				35						40				45
Lys	Asp	Arg	Arg	Asp	Phe	Arg	Phe	Pro	Gln	Glu	Met	Val	Lys	Gly



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	50		55		60									
Ser	Gln	Leu	Gln	Lys	Ala	His	Val	Met	Ser	Val	Leu	His	Glu	Met
	65		70		75									
Leu	Gln	Gln	Ile	Phe	Ser	Leu	Phe	His	Thr	Glu	Arg	Ser	Ser	Ala
	80		85		90									
Ala	Trp	Asn	Met	Thr	Leu	Leu	Asp	Gln	Leu	His	Thr	Gly	Leu	His
	95		100		105									
Gln	Gln	Leu	Gln	His	Leu	Glu	Thr	Cys	Leu	Leu	Gln	Val	Val	Gly
	110		115		120									
Glu	Gly	Glu	Ser	Ala	Gly	Ala	Ile	Ser	Ser	Pro	Ala	Leu	Thr	Leu
	125		130		135									
Arg	Arg	Tyr	Phe	Gln	Gly	Ile	Arg	Val	Tyr	Leu	Lys	Glu	Lys	Lys
	140		145		150									
Tyr	Ser	Asp	Cys	Ala	Trp	Glu	Val	Val	Arg	Met	Glu	Ile	Met	Lys
	155		160		165									
Ser	Leu	Phe	Leu	Ser	Thr	Asn	Met	Gln	Glu	Arg	Leu	Arg	Ser	Lys
	170													
Asp	Arg	Asp	Leu	Gly	Ser	Ser								

and the derivatives thereof N-glycolysed in amino acid position 78.

33. Interferon as claimed in claim 31, characterised in that the interferon as claimed in claim 32 contains in position 111 the amino acid Glu instead of Gly, and the derivatives thereof N-glycolysed in amino acid position 78.

34. Interferons as claimed in claims 30 to 33, characterised in that they contain the leader peptide of formula

Met Ala Leu Leu Phe Pro Leu Leu
Ala Ala Leu Val Met Thr Ser Tyr Ser Pro Val Gly Ser Leu Gly.

35. Process for preparing the interferon peptides as claimed in claims 26 to 34, characterised in that

a suitable host organism is transformed with genetic information coding for the interferon proteins as



claimed in claims 26 to 34,

this information is expressed in order to produce the corresponding interferon in the host organism and

the interferon peptide thus obtained is isolated from this host organism.

36. Process as claimed in claim 35, characterised in that the information is contained in each recombinant DNA molecule as claimed in claims 1 to 10.

37. Process as claimed in claim 36, characterised in that after transformation the host organism is defined according to one of the claims 15 to 20.

38. Process as claimed in claim 35, characterised in that the interferon is defined according to claims 32 to 34.

39. Process as claimed in claim 35, characterised in that E. coli HB101 is used as the host and a plasmid as claimed in claim 21, 22 or 23 is used as the plasmid.

40. Omega-interferon which can be prepared by the process as claimed in claim 35 or 39.

41. Mixture suitable for antitumour or antiviral treatment, containing in addition to one or more inert carriers and/or diluents an effective quantity of interferon peptide as claimed in one of claims 26 to 34.

42. Use of omega-interferon as claimed in claims 30 to 34 for preparing a mixture suitable for antitumour or antiviral treatment.

43. Process for preparing the plasmid pRHW10, characterised in that the DNA fragment of formula

HindIII	Sau3A	NcoI	
aAGCTTAAAG	ATGTGTGATC	TGCCTCAGAA	CCATGGCCTA CTTAGCAGGA 50
ACACCTTGGT	GCTTCTGCAC	CAAATGAGGA	GAATCTCCCC TTTCTTGTGT 100
CTCAAGGACA	GAAGAGACTT	CAGGTTCCCC	CAGGAGATGG TAAAAGGGAG 150



CCAGTTGCAG AAGGCCCATG TCATGTCTGT CCTCCATGAG ATGCTGCAGC 200

AGATCACACA TCTTTAagct t

Sau3A

HindIII

is inserted into the plasmid pER103, DSM no. 2773 (Fig. 6), at the HindIII site by ligase reaction, and the resulting plasmid is transformed for replication in E. coli HB 101 and cultivated.

44. Process for preparing the expression plasmid pRHW12, characterised in that the fragment of formula

c CAT GGC CTA CTT AGC AGG AAC ACC TTG 28

NcoI

GTG CTT CTG CAC CAA ATG AGG AGA ATC TCC CCT TTC TTG TGT CTC 73
AAG GAC AGA AGA GAC TTC AGG TTC CCC CAG GAG ATG GTA AAA GGG 118
AGC CAG TTG CAG AAG GCC CAT GTC ATG TCT GTC CTC CAT GAG ATG 163
CTG CAG CAG ATC TTC AGC CTC TTC CAC ACA GAG CGC TCC TCT GCT 208
GCC TGG AAC ATG ACC CTC CTA GAC CAA CTC CAC ACT GGA CTT CAT 253
CAG CAA CTG CAA CAC CTG GAG ACC TGC TTG CTG CAG GTA GTG GGA 298
GAA GGA GAA TCT GCT GGG GCA ATT AGC AGC CCT GCA CTG ACC TTG 343
AGG AGG TAC TTC CAG GGA ATC CGT GTC TAC CTG AAA GAG AAG AAA 388
TAC AGC GAC TGT GCC TGG GAA GTT GTC AGA ATG GAA ATC ATG AAA 433
TCC TTG TTC TTA TCA ACA AAC ATG CAA GAA AGA CTG AGA AGT AAA 478
GAT AGA GAC CTG GGC TCA TCT TGAAATGATTCTCATTGATTAATTTGCCATA 530
TAACACTTGCACATGTGACTCTGGTCAATTCAAAAGACTCTTATTTTCGGCTTTAATCAC 589
AGAATTGACTGAATTAGTTCTGCAAATACTTTGTCTGGTATATTAAGCCAGTATATGTTA 648
AAAAGACTTAGGTTTCAGGGGCATCAGTCCCTAAGATGTTATTTATTTTACTCATTTAT 707
TTATTCTTACATTTTATCATATTTATACTATTTATATTCTTATATAACAAATGTTTGCC 766
TTTACATTGTATTAAGATAACAAAACATGTTTCAGct 802

AluI

obtained by digesting the AvaII fragment of clone P9A2 (DSM no. 3004) with NcoI and AluI is inserted by ligase reaction into the larger fragment of plasmid pRHW10, which contains in the HindIII site of the plasmid pER103, DSM no. 2773, (Fig. 6), the DNA fragment of formula



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HindIII	Sau3A	NcoI	
aAGCTTAAAG	ATGTGTGATC	TGCCTCAGAA	CCATGGCCTA CTTAGCAGGA 50
ACACCTTGGT	GCTTCTGCAC	CAAATGAGGA	GAATCTCCCC TTTCTTGTGT 100
CTCAAGGACA	GAAGAGACTT	CAGGTTCCCC	CAGGAGATGG TAAAAGGGAG 150
CCAGTTGCAG	AAGGCCCATG	TCATGTCTGT	CCTCCATGAG ATGCTGCAGC 200
AGATCACACA TCTTTAagct t			
Sau3A	HindIII		

this larger fragment of the plasmid pRHW10 having been obtained by restriction endonuclease digestion with BamHI, filling the cutting sites with the aid of the Klenow fragment of DNA polymerase I and the 4 deoxynucleoside triphosphates and subsequent cutting with NcoI,

and the resulting plasmid is transformed and cultivated in E. coli HB 101 for replication.

45. Process for preparing the expression plasmid PHWR11, characterised in that the fragment of formula

c	CAT	GGC	CTA	CTT	AGC	AGG	AAC	ACC	TTG	28					
NcoI															
GTG	CTT	CTG	CAC	CAA	ATG	AGG	AGA	ATC	TCC	CCT	TTC	TTG	TGT	CTC	73
AAG	GAC	AGA	AGA	GAC	TTC	AGG	TTC	CCC	CAG	GAG	ATG	GTA	AAA	GGG	118
AGC	CAG	TTG	CAG	AAG	GCC	CAT	GTC	ATG	TCT	GTC	CTC	CAT	GAG	ATG	163
CTG	CAG	CAG	ATC	TTC	AGC	CTC	TTC	CAC	ACA	GAG	CGC	TCC	TCT	GCT	208
GCC	TGG	AAC	ATG	ACC	CTC	CTA	GAC	CAA	CTC	CAC	ACT	GGA	CTT	CAT	253
CAG	CAA	CTG	CAA	CAC	CTG	GAG	ACC	TGC	TTG	CTG	CAG	GTA	GTG	GGA	298
GAA	GGA	GAA	TCT	GCT	GAG	GCA	ATT	AGC	AGC	CCT	GCA	CTG	ACC	TTG	343
AGG	AGG	TAC	TTC	CAG	GGA	ATC	CGT	GTC	TAC	CTG	AAA	GAG	AAG	AAA	388
TAC	AGC	GAC	TGT	GCC	TGG	GAA	GTT	GTC	AGA	ATG	GAA	ATC	ATG	AAA	433
TCC	TTG	TTC	TTA	TCA	ACA	AAC	ATG	CAA	GAA	AGA	CTG	AGA	AGT	AAA	478
GAT	AGA	GAC	CTG	GGC	TCA	TCT	TGAAATGATTCTCATTGATTAATTTGCCATA	530							
TAACACTTGCACATGTGACTCTGGTCAATTCAAAAGACTCTTATTTGGGCTTTAATCAC	589														
AGAATTGACTGAATTAGTTCTGCAAATACTTTGTCTGGTATATTAAGCCAGTATATGTTA	648														



AAAAGACTTAGGTCAGGGGCATCAGTCCCTAAGATGTTATTTATTTTACTCATTTAT 707
TTATTCTTACATTTTATCATATTTATACTATTTATATTCTTATATAACAAATGTTTGCC 766
TTTACATTGTATTAAGATAACAAAACATGTTCAGct 802
AluI

obtained by digesting the *Ava*II fragment of clone E76E9 (DSM no. 3003) with *Nco*I and *Alu*I is inserted by ligase reaction into the larger fragment of plasmid pRHW10, which contains in the *Hind*III site of the plasmid pER103 (DSM no. 2773, see Fig. 6) the DNA fragment of formula

<i>Hind</i> III	<i>Sau</i> 3A	<i>Nco</i> I	
a <u>AGCTT</u> AAG	ATGTGT <u>GATC</u>	TGCCTCAGAA	<u>CCATGGCCTA</u> CTTAGCAGGA 50
ACACCTTGGT	GCTTCTGCAC	CAAATGAGGA	GAATCTCCCC TTTCTTGTGT 100
CTCAAGGACA	GAAGAGACTT	CAGGTTCCCC	CAGGAGATGG TAAAAGGGAG 150
CCAGTTGCAG	AAGGCCCATG	TCATGTCTGT	CCTCCATGAG ATGCTGCAGC 200
<u>AGATCACACA</u>	TCTTTA <u>agct</u>	t	
<i>Sau</i> 3A	<i>Hind</i> III		

this larger fragment of the plasmid pRHW10 having been obtained by restriction endonuclease digestion with *Bam*HI, filling the cutting sites with the aid of the Klenow fragment of DNA polymerase I and the 4 deoxynucleoside triphosphates and subsequent cutting with *Nco*I, and the resulting plasmid is transformed and cultivated in *E. coli* HB 101 for replication.

46. Synergistic mixture according to claim 41, characterised in that it additionally contains γ -interferon.

47. Synergistic mixture according to claim 41, characterised in that it additionally contains human tumour necrosis factor.