Volume 13 Number 13 1985 Nucleic Acids Research

A novel class of human type I interferons

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Received 12 April 1985; Revised and Accepted 11 June 1985

ABSTRACT

The screening of a cDNA library prepared from mRNA of Sendai virus induced Namalwa (human Burkitt's lymphoma) cells, using a human IFN-α2 DNA probe under conditions of low stringency, identified two weakly hybridizing clones containing sequences related to, but discernably different from those of the IFN-α class. Sequence and hybridization analysis of these cDNAs as well as expression in E.coli provided evidence that they encode proteins which have the characteristics of IFN type I but which are sufficiently diverged in sequence from both IFN-αs and IFN-β to suggest that they are representatives of a new and distinct class of interferons named interferon-omega. Hybridization of these sequences to genomic DNA reveals that this class contains at least four members.

INTRODUCTION

Previously identified type I interferons have been divided on the basis of their induction in different cell types and lack of serological cross-reactivity (1) into two main classes, leukocyte (IFN-α) and fibroblast (IFN-β) interferon. The complexity of the gene families encoding α- and β-interferons has been revealed using a combination of gene cloning and DNA hybridization techniques (2). In humans, the IFN-α class consists of at least 13 members which are more than 77% homologous in amino acid sequence (3-9). The IFN-β class consists of a single member, which has diverged considerably from members of the IFN-α class being only 30% homologous to them (10-13).

As part of a characterization of human type I interferons induced in Namalwa cells (14) after Sendai virus induction we have prepared a cDNA library in E.coli (15) and analysed the clones by low stringency hybridization with a hu-IFN-α2 DNA. In this report we describe the existence of two weakly hybridizing clones which are representatives of a new class of interferons named IFN-ω.

MATERIAL AND METHODS

Screening of the library

The construction of the cDNA library, the growth of the individual clones and the preparation of the nitrocellulose filters were described before (15). IFN-α2 DNA was excised from the plasmid pER33 (16) and contained the entire coding region for mature IFN-α2 plus 185 bp of 3'non-translated region. This DNA was nicktranslated to a specific activity of 100 $x10^6$ cpm/µg (17). The filters with the bound DNAs were hybridized in a solution containing 6xSSC (1xSSC = 150 mM NaCl, 15 mM trisodium citrate), 5xDenhardt's solution (18) and 0,1% SDS at 65°C for 18 hours. The washing was done 3 times 15 min at room temperature in 2xSSC, 0,1% SDS and 3 times 45 min in the same solution at 65°C for 45 min. The filters were dried and exposed to Kodak X-Omat S film using an intensifying screen at -70°C.

Nucleotide sequence determination

The cDNA inserts of the clones P9A2 and E76E9 were isolated by digestion with PstI, further cut with AluI, Sau3A or HaeIII and subcloned in M13mp9 (19) cut with the appropriate enzymes. Nucleotide sequencing was done according to Sanger et. al (20) using α-^{35}S-dATP and gradient gels (21). The sequences were assembled using the computer programmes originally developed by Staden (22), modified and improved by C.Pieler (this laboratory, unpublished) for a Cyber CDC 170 (Standard Fortran 77).

Genomic Southern blot hybridization

Total DNA from Namalwa cells was isolated using the method of Blin et. al (23). 10 µg DNA/lane were digested with EcoRI, HindIII, BamHI, SphI, PstI or ClaI under conditions specified by the supplier (New England Biolabs). The DNA was separated on a 1% agarose gel and transfered to a nitrocellulose filter according to Southern (24). The filter was cut in half, baked at 80°C for 2 hours and prehybridized at 65°C for 4 hours in 6x SSC, 5x Denhardt's solution, 0,1% SDS. One half of the filter was hybridized with $10x10^6$ cpm of nick-translated IFN-α2 DNA (the same fragment as used for screening) and the other half with $10x10^6$ cpm nick-translated Sau3A-AluI fragment of the P9A2 cDNA insert (628bp, see Fig.2b) again in 6x SSC, 5x Denhardt's solution, 0,1% SDS at 65°C for 18 hours. The filters were washed with 2x SSC, 0,01% SDS 3 times 15 min at room temperature and with 0,2x SSC, 0,01% SDS 3 times 45 min at 65°C. The filters were dried and exposed to Kodak X-Omat S X-ray film using an intesifying screen at -70°C.

DNA preparations

Plasmid DNAs were prepared by the cleared lysate method (25) and were purified by CsCl-density centrifugation. Small scale plasmid preparations were done according to Birnboim et. al. (26). DNA restriction fragments were recovered from 1,0% or 1,4% agarose gels by electroelution followed by Elutip column chromatography (Schleicher and Schuell) and ethanol precipitation.

Construction of the expression plasmid pRHW12

The expression vector pER103 and the preparation of the HindIII-Sau3A adaptor containing the start codon ATG and the codon for the first amino acid of mature α-IFNs (TGT) were described by Rastl-Dworkin et.al.(16). T_4-polynucleotide kinase, DNA polymerase I-large fragment (both from Boehringer-Mannheim) and T_4-DNA ligase (NEN) were used following standard recipes (27). The P9A2-cDNA insert was isolated together with some flanking regions from pBR322 by cutting with AvaII. The DNA was modified as indicated in fig.3. E.coli HB101 was transformed with the resulting expression plasmid pRHW12. The plasmid DNA from one of the colonies was isolated and the IFN-gene containing EcoRI-BamHI-fragment sequenced to verify the construction.

Interferon assay

E.coli HB101 transformed with pRHW12 was grown in M9 medium (28) supplemented with 0,1 mM $CaCl_2$, 1 mM $MgSO_4$, 1,1% glucose, 2,1% casamino acids (Merck), 20 µg/ml cysteine, 1 µg/ml thiamine.HCl, 100 µg/ml ampicillin and 20 µg/ml indole acrylic acid to an A_{600} of about 2. The cells were pelleted by centrifugation, resuspended in 0,1 vol 50 mM Tris/Cl pH=7,6, 30 mM NaCl and broken up by sonication (MSE 100 Watt Ultrasonic Disintegrator, max. output at 20 kHz for 2 times 0,5 min.). The cell debris were removed by centrifugation and the supernatant sterile filtered. This supernatant was used either directly or brought to pH=2 by the addition of HCl and incubated 16 hours at 4°C. The pH was then readjusted to 7,4. The supernatant was assayed for interferon activity with the CPE reduction assay using A549 (human lung carcinoma) cells and encephalomyocarditis virus (29). The interferon activity is expressed as units relative to the NIH leukocyte interferon standard GO-23-901-527.

RESULTS

Screening of a cDNA library

As part of a characterization of human type I interferons induced in

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                                                                  Leu Gly
                                                                T CTG GGC      7

   1               5                  10                  15                  20
 Cys Asp Leu Pro Gln Asn His Gly Leu Leu Ser Arg Asn Thr Leu Val Leu Leu His Gln
 TGT GAT CTG CCT CAG AAC CAT GGC CTA CTT AGC AGG AAC ACC TTG GTG CTT CTG CAC CAA    67

                  25                  30                  35                  40
 Met Arg Arg Ile Ser Pro Phe Leu Cys Leu Lys Asp Arg Arg Asp Phe Arg Phe Pro Gln
 ATG AGG AGA ATC TCC CCT TTC TTG TGT CTC AAG GAC AGA AGA GAC TTC AGG TTC CCC CAG   127

                  45                  50                  55                  60
 Glu Met Val Lys Gly Ser Gln Leu Gln Lys Ala His Val Met Ser Val Leu His Glu Met
 GAG ATG GTA AAA GGG AGC CAG TTG CAG AAG GCC CAT GTC ATG TCT GTC CTC CAT GAG ATG   187

                  65                  70                  75                  80
 Leu Gln Gln Ile Phe Ser Leu Phe His Thr Glu Arg Ser Ser Ala Ala Trp Asn Met Thr
 CTG CAG CAG ATC TTC AGC CTC TTC CAC ACA GAG CGC TCC TCT GCT GCC TGG AAC ATG ACC   247

                  85                  90                  95                 100
 Leu Leu Asp Gln Leu His Thr Gly Leu His Gln Gln Leu Gln His Leu Glu Thr Cys Leu
 CTC CTA GAC CAA CTC CAC ACT GGA CTT CAT CAG CAA CTG CAA CAC CTG GAG ACC TGC TTG   307

                 105                 110 Glu             115                 120
 Leu Gln Val Val Gly Glu Gly Glu Ser Ala Gly Ala Ile Ser Ser Pro Ala Leu Thr Leu
 CTG CAG GTA GTG GGA GAA GGA GAA TCT GCT GGG GCA ATT AGC AGC CCT GCA CTG ACC TTG   367
                                         A
                 125                 130                 135                 140
 Arg Arg Tyr Phe Gln Gly Ile Arg Val Tyr Leu Lys Glu Lys Lys Tyr Ser Asp Cys Ala
 AGG AGG TAC TTC CAG GGA ATC CGT GTC TAC CTG AAA GAG AAG AAA TAC AGC GAC TGT GCC   427

                 145                 150                 155                 160
 Trp Glu Val Val Arg Met Glu Ile Met Lys Ser Leu Phe Leu Ser Thr Asn Met Gln Glu
 TGG GAA GTT GTC AGA ATG GAA ATC ATG AAA TCC TTG TTC TTA TCA ACA AAC ATG CAA GAA   487

                 165                 170
 Arg Leu Arg Ser Lys Asp Arg Asp Leu Gly Ser Ser  *
 AGA CTG AGA AGT AAA GAT AGA GAC CTG GGC TCA TCT TGA AATGATTCTCATTGATTAATTTGCCAT   553

 ATAACACTTGCACATGTGACTCTGGTCAATTCAAAAGACTCTTATTTCGGCTTTAATCACAGAATTGACTGAATTAGTT   632

 CTGCAAATACTTTGTCGGTATATTAAGCCAGTATATGTTAAAAAGACTTAGGTTCAGGGGCATCAGTCCCTAAGATGTT   711

 ATTTATTTTTACTCATTTATTTATTCTTACATTTTATCATATTTATACTATTTATATTCTTATATAACAAATGTTTGCC   790

 TTTACATTGTATTAAGATAACAAAACATGTTCAGCTTTCCATTTGGTTAAATATTGTATTTTGTTATTTATTAAATTAT   869

 TTTCAAAC - poly A                                                               877
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Fig.1. Nucleotide and derived protein sequence of the cDNA clone P9A2. The triplets coding for cysteins as well as the potential N-glycosylation site are boxed. The poly-adenylation signal (ATTAAA) is underlined. The dG/dC tails produced during the course of cloning are omitted. -polyA indicates 22 dA-residues. The E76E9 cDNA starts one nucleotide earlier (CTCTG...) and ends three nucleotides before the ATTAAA sequence (...GTTAT). The single nucleotide difference at triplet 111 leading to an amino acid change is also boxed.

Namalwa cells after Sendai virus induction we have prepared a cDNA library in E.coli (15) and analysed the clones as follows. mRNA from Sendai virus induced Namalwa cells was primed with the synthetic tridecamer

Table 1. Nucleotide and amino acid differences among type I interferons.

	IFN-αA	IFN-αB	IFN-αC	IFN-αD	IFN-αF	IFN-αG	IFN-αH	IFN-αJ	IFN-αL	IFN-αN	IFN-β	IFN-ω
IFN-αA	-------	31 (19)	31 (19)	29 (18)	30 (18)	25 (15)	29 (18)	34 (21)	35 (21)	30 (18)	109+5 (70)	63+6 (42)
IFN-αB	68 (13)	-------	32 (19)	38 (23)	31 (19)	29 (18)	30 (18)	35 (21)	35 (21)	29 (18)	113+5 (72)	69+6 (45)
IFN-αC	59 (11)	65 (13)	-------	32 (19)	18 (11)	27 (16)	24 (15)	14 (8)	4 (2)	24 (15)	110+5 (70)	67+6 (44)
IFN-αD	60 (12)	72 (14)	63 (12)	-------	28 (17)	24 (15)	32 (19)	37 (22)	35 (21)	36 (22)	112+5 (71)	70+6 (46)
IFN-αF	64 (12)	66 (13)	42 (8)	59 (11)	-------	20 (12)	27 (16)	23 (14)	22 (13)	28 (17)	112+5 (71)	72+6 (47)
IFN-αG	55 (11)	65 (13)	52 (10)	56 (11)	53 (10)	-------	24 (15)	31 (19)	30 (18)	27 (16)	109+5 (70)	68+6 (45)
IFN-αH	53 (10)	66 (13)	42 (8)	58 (11)	57 (11)	46 (9)	-------	27 (16)	27 (16)	23 (14)	108+5 (69)	71+6 (46)
IFN-αJ	61 (12)	69 (13)	20 (4)	69 (13)	45 (9)	54 (10)	43 (8)	-------	16 (10)	26 (16)	110+5 (70)	71+6 (46)
IFN-αL	61 (12)	67 (13)	4 (1)	65 (13)	44 (9)	53 (10)	43 (8)	20 (4)	-------	28 (17)	111+5 (71)	68+6 (45)
IFN-αN	59 (11)	64 (12)	36 (7)	67 (13)	54 (11)	52 (10)	43 (8)	38 (7)	38 (7)	-------	109+5 (70)	71+6 (46)
IFN-β	275 (53)	279 (54)	279 (54)	283 (55)	275 (53)	276 (52)	275 (53)	278 (54)	281 (54)	269 (52)	----------	111+11(74)
IFN-ω	148 (29)	156 (30)	152 (29)	154 (30)	160 (31)	147 (28)	155 (30)	156 (30)	153 (30)	155 (30)	270 (52)	---------

Amino acid differences

Nucleotide differences

The first number represents the absolute value of differences followed by the percentage in brackets. The extra numbers in the IFN-β and IFN-ω columns represent the numbers of nonaligned amino acids which are already included in the percentage (one amino acid contributes 0,6%).

d(CCTTCTGGAACAG) complementary to many IFN-αs and the IFN-β mRNA (30). cDNA was prepared by standard techniques and used to screen part of the library. One positively reacting clone was confirmed by nucleotide sequencing to contain an IFN-α2 specific cDNA. This IFN-α2 cDNA was used to screen the entire library under low stringency conditions (31). In addition to the strong signals from clones containing IFN-α cDNAs, two weakly hybridizing clones were observed. These were further characterized by Southern hybridization (24) and found to strongly hybridize to each other and to weakly hybridize to purified IFN-α2 cDNA. Restriction analysis of the two clones named P9A2 and E76E9 suggested that although they differed in size by approximately 40 nucleotides they correspond to mRNAs of very similar sequence.

Nucleotide sequencing

The complete nucleotide sequences of both cDNAs were determined using the dideoxy chain termination method (20) and the results obtained are shown in Fig.1. The clones contained almost identical sequences differing only at a single location (P9A2 position 339) and in the size of the insert, i.e. 899 nucleotides for P9A2, 858 nucleotides for E76E9. Both cDNAs contain a single open reading frame which encodes the protein sequences illustrated in Fig.1. P9A2 cDNA contains a stretch of 22 dAs commencing 352 bases after the stop codon of the major open reading frame (-polyA in Fig.1) preceded by an ATTAAA sequence suggesting that this corresponds to the authentic 3'terminus of the mRNA (32,6).

Sequence analysis

The encoded proteins can be aligned with IFN-αs on the basis of the

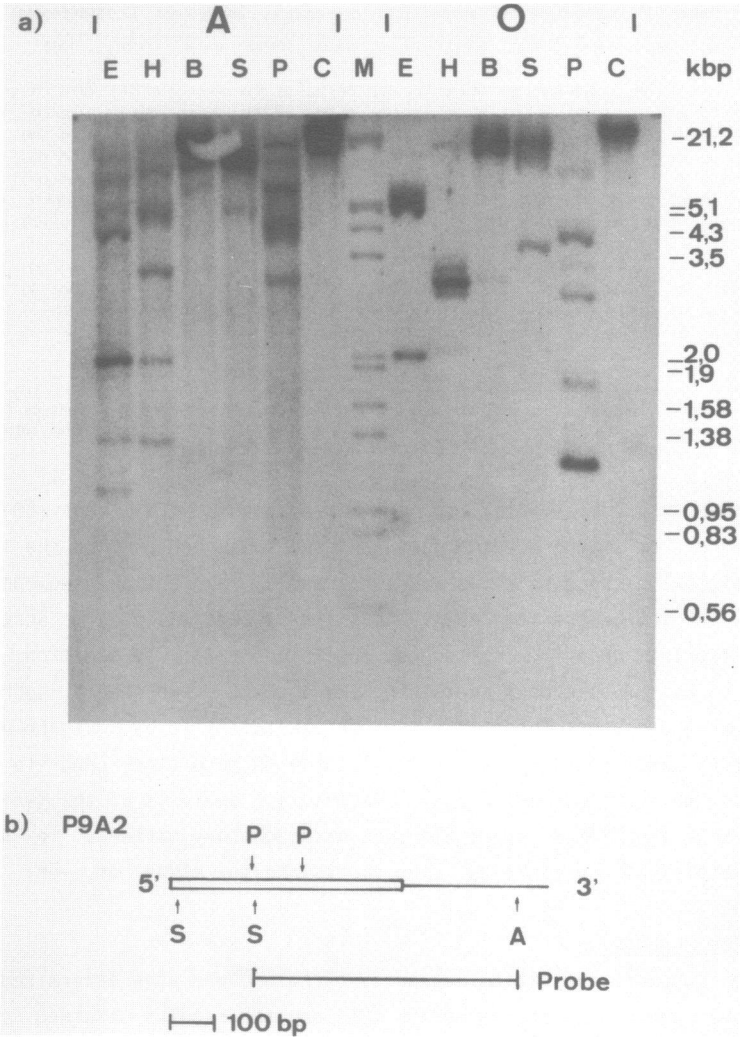

Fig.2. a) Southern blot analysis of genomic DNA. The DNA was digested with different enzymes (E=EcoRI, H=HindIII, B=BamHI, S=SphI, P=PstI, C=ClaI). Half of the filter ("A") was hybridized with radiolabelled IFN-α2 DNA, the other half ("O") with part of the P9A2-cDNA. M=size marker. b) Restriction map of P9A2 cDNA indicating the fragment used for hybridization.

Cys-Asp-Leu-Pro start peptide of the mature interferons (6), with IFN-β on the basis of the potential N-glycosylation site (Asn-Met-Thr at position 78 to 80 in P9A2- and E76E9 protein sequence and Asn-Glu-Thr at positions 80 to 82 in IFN-β (10)) and with both IFN-αs and IFN-β on the basis of conserva-

tion of the essential cysteinyl-residues at positions 29 and 139 in IFN-αs and positions 31 and 141 in IFN-β (33). It should be noted that the new sequences encode a mature protein being 172 amino acids in length.

Based on this arrangement the corresponding nucleotide sequences of IFN-αs, IFN-β and the new sequences (named IFN-ω in table 1) were pairwisely aligned and the differences counted. The result is shown in table 1. Differences among individual IFN-α DNAs range between 0 (IFN-αI and IFN-αI3 (34)) and 14% (IFN-αB and IFN-αD), whereas the new DNA sequences differ from the published IFN-α DNA sequences by at least 28 and up to 31%. All IFN-α DNAs as well as the new sequences differ by more than 50% from the IFN-β DNA sequence (lower left part in Table 1). An even more drastic result is seen when amino acid sequences of mature IFNs are compared. Members of the IFN-α class vary up to 23% whereas pairwise comparison of the new protein with individual members of the IFN-α proteins reveals differences of at least 42 and up to 47%. Again the divergence of both IFN-α and the P9A2 or E76E9 derived proteins from the IFN-β protein is about equal, i.e. 70% (top right part in Table 1).

Total DNA blotting analysis

Completely digested Namalwa DNA was probed after agarose gel electrophoresis and Southern transfer under more stringent conditions not allowing for cross hybridization between the new sequences and those of IFN-αs and IFN-β. Using part of P9A2 cDNA as the radiolabelled probe reveals multiple bands (Fig.2a). This set of bands was different from the one hybridizing with the IFN-α2 probe. Only one band of the PstI pattern generated with the P9A2 probe corresponds to the authentic P9A2 gene since this DNA could not detect sequences 5' to the first internal PstI site. In addition sequences shorter than 500 base pairs run off the gel resulting in loss of the internal 120 bp long PstI fragment (Fig. 2b). Therefore the number of fragments of PstI restricted DNA hybridizing to the P9A2 probe leads to the estimation of at least four members of the new IFN class.

Expression of the P9A2 cDNA-sequence in E.coli

In order to show that the new sequence encodes a protein with antiviral activity the cDNA sequence contained in the plasmid P9A2 was modified and cloned in the expression vector pER103 (16). The procedure is outlined in Fig.3. The cDNA sequence of the clone P9A2 starting with the codon for cystein was placed under the control of the trp-promoter (Serratia marcescens) and is preceded by a ribosomal binding site and a translation start codon. The resulting plasmid was named pRHW12. E.coli HB101 transformed with this plasmid was grown in medium containing indole acrylic acid. Cell

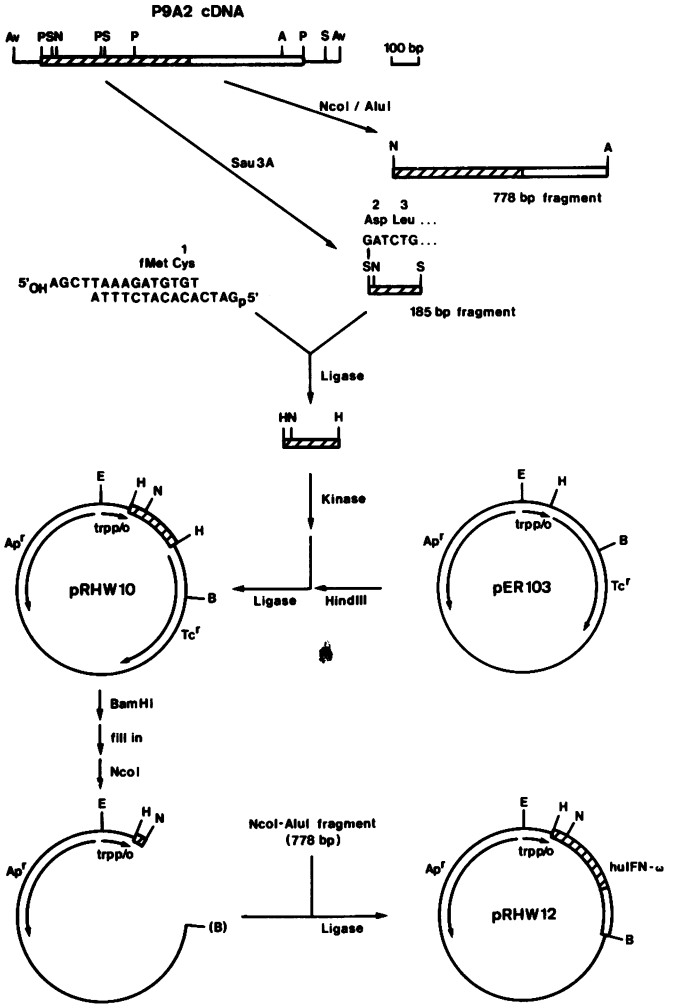

Fig.3. Strategy for the construction of the expression plasmid pRHW12. A=AluI, Av=AvaII, B=BamHI, H=HindIII, N=NcoI, P=PstI and S=Sau3A. The plasmids are not drawn to scale.

extracts were measured for interferon activity using the CPE reduction assay (29). We found $1x10^5$ units per liter culture with or without pH=2 treatment of the extract.

DISCUSSION

We detected sequences in a cDNA library resembling authentic interferon

cDNAs. Expression of the corresponding mRNAs at a detectable level occurs only after virus induction (C.Pieler, this laboratory, in preparation). The sequences encode proteins which after expression in E.coli exhibit stability at pH=2 and antiviral activity.

These proteins are 172 amino acids long and contain a potential N-glycosylation site at a location corresponding to the one in IFN-β. The amino acid comparison between the P9A2 or E76E9 encoded proteins and individual proteins of the IFN-α class reveals differences in the range between 41 to 47%. The difference to IFN-β is about 70%. Recommendations for the nomenclature of IFNs suggest that interferons should be considered as members of a new class "if there is a difference in more than about 50% of the encoded amino acid residues" (35). Although by accepting this rigidly the new interferon would be classified as an IFN-α, we would like to propose that the proteins encoded by the clones P9A2 and E76E9 should be regarded as members of a new class designated IFN-ω.

Southern blot analysis of total DNA shows the existence of at least four members belonging to this new class of IFNs. It is not yet possible to decide whether the cDNAs of P9A2 and E76E9 are derived from the transcripts of two distinct genes or two allelic forms of one gene or whether the nucleotide difference at P9A2 position 339 is due to an error of the reverse transcriptase during the course of the cloning experiment (36).

Further work to identify the other members of the IFN-ω class as well as characterization of the proteins coded for by the cDNA clones P9A2 and E76E9 is in progress.

ACKNOWLEDGEMENTS

We thank Dr. P. Meindl for help with sequencing work, H. Wocelka and B. Demeties for technical assistance, Drs. G.Bodo and E.Falkner for help with growing E.coli and the CPE reduction assay and Drs. J.W.Almond and G. Adolf for valuable discussions and help in preparing this manuscript.

NOTE

After completion of this manuscript D.J.Capon et al. (Molec. Cell. Biol. 5, 768-779 (1985)) published the finding of two distinct families of human and bovine Interferon-α genes. They subdivided the IFN-α class into two subfamilies: class I and class II IFN-α genes. The comparison of the HuIFN-$\alpha_{II}1$ gene with the P9A2 and E76E9 sequences reveals two differences: HuIFN-$\alpha_{II}1$ contains a GAA-triplet coding for Glu-88 whereas our sequences

were confirmed by careful rechecking of the sequencing gels to code for Gly-88 (GGA). Secondly the 3'nontranslated region of IFN-$\alpha_{II}1$ reads CATAATAAC starting at position 25 after the stop codon whereas the P9A2- and E76E9 cDNAs contain CATATAAC at this location. The HuIFN-$\alpha_{II}1$ gene contains the GGG triplet at amino acid position 111 confirming the sequence of the P9A2 cDNA. These differences could be due to allelic variations since the genes were isolated from different sources, i.e. fetal liver or Namalwa cells.

We would suggest based on the conclusions described in the discussion part and for clarity reasons to use the name IFN-ω instead of subdividing the α-IFNs in class I and class II IFN-αs.

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