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The Woodchuck Interferon-α System: Cloning, Family Description, and Biologic Activity

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Interferon-α (IFN-α) is a key element in the defense against viral infection because, in addition to a direct antiviral effect, it exhibits potent immunostimulatory activity. To investigate the function of this cytokine in the woodchuck model of chronic hepatitis B, the woodchuck IFN-α gene (IFNA) family was cloned and examined. The data indicate that this is a multigenic family from which 12 IFNA functional sequences and four pseudogene sequences were isolated. The overall identity of the amino acid sequence among the members of the woodchuck IFN-α family is 85%, and the identity with the IFN-α family from other species such as mice and humans is 50%. The analysis of hepatic expression of IFNA genes showed that wIFNA5a was the subtype transcribed preferentially in the woodchuck liver. The wIFNA genes transcribed in the liver were tested in an eukaryotic expression system and were found to enhance 2–5-oligoadenylate synthetase (2–5-OAS) mRNA levels and to posses a potent antiviral activity. Cloning of woodchuck IFNA genes will allow testing diverse forms of IFN-α delivery as well as different combination therapies in woodchuck hepatitis virus infection, thus providing useful information for the design of new strategies for the treatment of patients with chronic hepatitis B. ***J. Med. Virol. 68:424–432, 2002.*** © 2002 Wiley-Liss, Inc.

KEY WORDS: antiviral; viral hepatitis; 2–5-oligoadenylate synthetase

INTRODUCTION

The interferon-α (IFN-α) are members of the type I interferons, a group that includes the IFN-α, IFN-β, IFN-ω, and IFN-τ [Roberts et al., 1998]. Type I interferons are produced by a great diversity of cells in response to viral infections. The IFN-α system is a family of related proteins encoded by distinct IFNA genes. Although the IFN-α were first identified by their antiviral properties [Issacs and Lindenman, 1957], they have been shown to display a wide variety of biological effects, including participation in the cytokine network, which regulates the immune system [Biron, 2001]. The biological response requires direct interaction of the IFN-α molecule with the type I receptor complex, composed of two units that undergo rapid ligand-dependent tyrosine phosphorylation, followed by induction of at least 30 genes, including 2–5-OAS and Mx protein [David, 1995; Der et al., 1998]. Owing to its antiviral, immunoregulatory, and antiproliferative activities [Issacs and Lindenman, 1957; Fleischmann et al., 1998; Biron, 2001], the recombinant IFN-α has been approved for use in a number of clinical conditions, including chronic hepatitis B, chronic hepatitis C, malignant melanoma, hairy cell leukemia, and acquired immunodeficiency syndrome (AIDS)-related Kaposi's sarcoma.

Together with lamivudine, IFN-α constitutes the current treatment for persistent hepatitis B virus (HBV) infection, which affects more than 350 million people, often leading to chronic hepatitis, cirrhosis, and liver cancer [Maddrey, 2000]. However, the response rate to recombinant IFN-α is only around 30%; it also has unwanted side effects, such as depression, nausea, fever, fatigue, headaches, and muscle aches. To overcome these problems, new antivirals [Malaguarnera et al., 2001] and new methods of IFN-α delivery are under development. New forms of IFN-α administration include the use of biopolymers [Yamagata et al., 2000], stabilizing ligands [Zeuzem et al., 2000], and gene therapy [Protzer et al., 1999; Aurisicchio et al.,

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2000]. Testing the efficacy of these new treatments requires systematic in vivo studies in animal models.

Woodchuck hepatitis virus (WHV) infection in woodchuck (*Marmota monax*) has been shown to be a useful model for the study of the pathogenesis, prevention, and treatment of HBV infection [Roggendorf and Tolle, 1995; Menne and Tennant, 1999]. The replicative cycle of WHV, the genomic organization, and the proteins encoded by WHV DNA are very similar to those of HBV [Feitelson et al., 1981; Galibert et al., 1982]. As in humans, woodchucks exposed perinatally to WHV develop chronic hepatitis; this condition, similar to HBV infection, may progress to hepatocellular carcinoma. Thus, WHV infection represents a useful experimental model with which to examine the efficacy of antiviral strategies within the context of chronic illness.

The IFN-α family of woodchucks was cloned and characterized to provide a tool with which to test the antiviral effect of new forms of IFN-α delivery and to improve the efficacy of current therapies. The woodchuck IFN-α family was found to be composed of at least 12 different IFNA sequences and 4 pseudogenes, sharing an overall homology of 85% at the amino acid level. The hepatic expression of IFN-α subtypes was examined; it was found that wIFNA6a, wIFNA1b, and wIFNA5a sequences are expressed in liver tissue, with wIFNA5a the most common in this organ. These three IFN-α subtypes possess antiviral activity and induce 2–5-OAS expression when transfected into a woodchuck hepatoma cell line. Because wIFNA5a combines the property of being the natural IFN-α expressed in the liver with the fact of having a potent antiviral effect, this particular subtype appears to be the most appropriate for evaluation of new ways of IFN-α therapy in woodchucks.

MATERIALS AND METHODS

Animals and Cells

Six woodchucks (purchased from Northeastern Wildlife, Ithaca, NY), handled according to the guidelines of the institution (Centro de Investigación Farmacobiológica Aplicada, Pamplona, Spain), were used. Blood was collected from the saphenous vein of the hind legs, under anesthesia. Liver samples were obtained from woodchuck biopsies stored in the laboratory. WCH17 cells were obtained from the American Type Cell Culture (ATCC) (CRL2082) and were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS), under standard conditions.

Construction and Screening of a cDNA Library

Peripheral blood mononuclear cell (PBMCs) were isolated from a woodchuck as described previously [Hervas-Stubbs et al., 1997] and cultured at 37°C and 5% CO_2 in RPMI-1640 medium supplemented with 0.1 mg/ml of polyinosinic-polycytidylic acid (pIpC) plus 250 ml/ml of diethylaminoethyl dextran (DEAE-D) for 1 hr [Kaplan et al., 1987]. After incubation, cells were washed twice with phosphate-buffered saline (PBS) and incubated for 5 hr in RPMI 1640 with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2×10^{-5} M β-mercaptoethanol. Cells were harvested and total RNA extracted with the Ultraspec II system (Biotex Laboratories, Houston, TX). mRNA was purified by an mRNA isolation kit (Boehringer GmBH, Mannheim, Germany) according to the manufacturer's instructions.

The cDNA library from this mRNA was constructed, using the SMART cDNA library construction kit (Clontech, Palo Alto, CA), according to the manufacturer's instructions. A full-length human IFN-α5 cDNA probe obtained by polymerase chain reaction (PCR) amplification was labeled with ^{32}P and was used to screen the library under nonstringent conditions. Positive recombinant clones were isolated by repeated plaque purification and conversion of the λTriplEx2 recombinant clones to pTriplEx2 was undertaken in *Escherichia coli* strain BM25.8 according to the manufacturer's instructions. Finally, plasmids were isolated and sequenced following standard procedures.

Cloning of wIFN-α from Genomic DNA

Genomic DNA was extracted from liver sample of two woodchucks by DNA isolation kit (Qiagen, Hilden, Germany). Amplification of genomic wIFN-α was carried out by PCR in a final volume of 50 μl with 1× buffer, 0.2 μM of each dNTPs, 500 nM primers, 200 ng of gDNA, and 1 U/μl Taq DNA polymerase. PCR conditions were as follows: one initial denaturalization step at 94°C for 1 min, followed by 30 cycles at 94°C for 15 sec, 55°C for 15 sec, and 72°C for 40 sec, and a single final extension at 72°C for 1 min. Oligonucleotide primers IFNE1 (5′-CAGCAGCATCTTCAAGATCC-3′) and IFNE2 (5′-GAGTGTGGTAAGGTGTGTTC-3′) were designed from conserved sequences of 5′ and 3′ noncoding regions of the wIFNA clones isolated previously. A total of four independent PCRs were conducted, three from one woodchuck and another from a second woodchuck. PCR products were analyzed by agarose gel electrophoresis and DNA bands of expected size were isolated using Concert Rapid Gel Extraction System (Gibco-BRL, Eggestein-Leopoldshafen, Germany) and cloned into pCR2.1TOPO (Invitrogen, Groningen, The Netherlands). Ten clones were selected, and plasmids were isolated and sequenced.

Southern Blot Analysis

In this study, 20 μg of genomic DNA was digested to completion with *Bam*H I and *Hin*d III. DNA fragments were separated by electrophoresis on 0.8% agarose gels and transferred to a Hybond-N membranes (Amersham, Buckinghamshire, UK) and ultraviolet (UV) crosslinked. A DNA fragment containing the entire wIFNA1a open reading frame (ORF) was amplified by PCR with primers IFNE1 and IFNE2 and used as a

hybridation probe after labeling by the method of random primers with [α-^{32}P]dATP. Hybridization was conducted in ULTRAhyb solution (Ambion, Austin, TX), overnight at 42°C. The filter was washed twice in 2× sodium chloride/sodium citrate (SSC)/0.1× sodium dodecyl sulfate (SDS) at 42°C, twice in 0.1× SSC/0.1× SDS at 42°C, and three times in 0.1× SSC/0.1× SDS at room temperature and then exposed to X-ray film. Densitometry with the software Quantity one 4.2.1 (Bio-Rad, Hercules, CA) was carried out.

Analysis of wIFN-α Expressed in Woodchuck Liver

Needle biopsies were obtained from the liver of three woodchucks. Biopsies were homogenized and RNA was extracted using the Ultraspec-II system. In this study, 800 ng of total RNA was treated with 0.1 U/μl of DNase I (Gibco-BRL, Merelbeke, Belgium) in 1× DNase I reaction at room temperature for 15 min, and the DNase I was inactivated by addition of 1 μl of 25 mM EDTA solution and heat 10 min at 65°C. A reverse transcription (RT) reaction was performed in a final volume of 40 μl containing 800 ng of total RNA and 1× RT buffer, 5 mM dNTPs, 20 μM random hexamers, 0.4 U/μl RNase inhibitor and 32 U/μl MuLV reverse transcriptase. PCR was undertaken in 50 μl with 10 μl RT reaction (containing cDNA corresponding to 200 ng of the original RNA), 1× PCR buffer, 0.2 mM dNTPs, 1 U/μl of Taq DNA polymerase and 500 nM of each primer IFNE1 and IFNE2. PCR products were purified and cloned into pCR2.1TOPO (Invitrogen, Groningen, The Netherlands). Nine clones from woodchuck A, 10 clones from woodchuck B, and 8 clones of woodchuck C livers were analyzed as previously described. To rule out any possible bias due to the primers used for PCR, genomic DNA was analyzed with the same pair of primers and the same PCR conditions.

2–5-OAS Stimulation Assay

wIFNA sequences expressed in the liver were subcloned into pcDNA3.1V5HisTOPO vector (Invitrogen, Groningen, Germany) as well as the human IFNA5 gene. The orientation of the cDNA insert was analyzed by restriction enzyme analysis; clones with the insert in sense orientation were selected. In this study, 1 μg of these plasmids was cotransfected, using polyethylenimine (PEI) with 1 μg of pLucWRE, a plasmid that codifies luciferase and that contains the woodchuck posttranscriptional regulatory element (WPRE) of woodchuck hepatitis virus. Briefly, 22-kDa linear PEI (kindly provided by Dr. Jean Paul Behr) was used at an nitrogen to phosphate (N/P) ratio of 10 and using NaCl 150 mM as the vehicle; 5×10^5 WCH17 cells were plated in six-well plates and 24 hr later were cotransfected. After 24 hr, supernatant were collected and stored at −80°C. Cells were tripsinized and samples corresponding to each well were separated in two tubes, one for RNA extraction and the other for luciferase assay. Tubes were centrifuged at 830*g* for 5 min and supernatant was discarded. Pellets for luciferase assay were lysed in 100 μl 1× lysis buffer and frozen at −80°C for 5 min. After centrifugation at 14,000 rpm for 1 min, 5 μl of sample was added to 15 μl of 1× lysis buffer, and luciferase activity was measured using luciferase assay reagent (Promega, Madison, WI) in a luminometer; 15 μl of the sample was used to measure total protein with the Bradford method.

Total RNA of pellets for RNA extraction was isolated with the Ultraspec-II system following the manufacturer's instructions and finally diluted in 30 μl of RNase free water. RT was carried out in a final volume of 105 μl containing 840 ng of RNA, 1× RT buffer, 5.5 mM $MgCl_2$, 2 mM dNTPs, 2.5 μM random hexamers, 0.4 U/μl RNase inhibitor, and 1.25 U/μl MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). The parameters used were 10 min 25°C, 30 min 48°C, and 5 min 95°C. Primers and TaqMan probes (Applied Biosystems) for 2–5-OAS (sense: 5′-TTTTGATGCCCTGGGTCAGT-3′, antisense: 5′-AGGTGCACTCCTCGATGAGC-3′, probes: 5′-ACAGACCCAGCCCCCAGATCTATGT-3′) and β-actin (sense: 5′-TCACCCACACTGTGCCCATCTACGA-3′, antisense: 5′-CAGCGGAACCGCTCATTGCCAATGG-3′, probes: 5′-ATGCCCTCCCCCATGCCATCCTGCGT-3′) were designed according to the published cDNA sequences of woodchuck genes, using Primer-Express software. The real-time PCR reaction was carried out in 25 μl with 12.5 μl of RT reaction (containing cDNA corresponding to 100 ng of the original RNA), 1× TaqMan buffer, 0.4 mM of each dNTP, 0.01 U/μl of Amperase, 0.05 U/μl of Biotaq DNA polymerase (Bioline, London, UK), 300 nM of each primer, and 200 nM of the probe. PCR was performed according to the following parameters: 2 min 50°C for amperase digestion, initial denaturation for 15 sec at 94°C, and 45 cycles of 0.1 sec at 95°C, and hybridation/elongation of 20 sec at 60°C. The fluorescence signal delivered during PCR amplification was monitored using the LightCycler System (Roche Diagnostics, Basel, Switzerland). The number of copies of 2–5-OAS and β-actin was determined by interpolation, using external RNA standards. For preparation of the standard curves, 2–5-OAS and β-actin fragments were cloned into PCR2.1TOPO (Invitrogen, Groningen, Germany), containing a T7 promoter and in vitro transcribed with T7 RNA polymerase. The RNA concentration was estimated by optical density, and the copy number was calculated from the concentration, mean molecular weight of nucleotides (330 g/mol), and RNA length. The activity of woodchuck interferon is shown as copies of 2–5-OAS per 10^9 copies of β-actin normalized by RLU per mg of total protein.

Cytopathic Effect (CPE) Reduction Assay

The antiviral activity of wIFN-α present in the medium from cells cotransfected with wIFN-α and luciferase expressing plasmids were assayed in a cytopathic effect reduction assay using woodchuck WCH17 cells challenged with encephalomyocarditis (EMC) virus in 96-well microtiter plates.

Briefly, 1.9×10^4 WCH17 cells per well were plated in a 96-well plate and stimulated with threefold serial dilutions of medium from cotransfected cells. After 24 hr, cells were challenged with EMC virus at a multiplicity of infection 100:1; 24 hr later, cells were washed three times with PBS and stained with crystal violet solution. After washing the excess of crystal violet, 200 μl per plate of 1% SDS was added, and absorbance was measured at 540 nm. Because a woodchuck reference IFN-α is not available, the activity of woodchuck interferon is shown as the reciprocal of the dilution leading to 50% CPE.

Statistical Analysis and Phylogenetic Analysis

Statistical analysis for the CPE reduction assay and 2–5-OAS stimulation assay was carried out with the Kruskal-Wallis test and the Mann-Whitney U-test. Differences were considered significant at a P value of <0.05. Phylogenetic analysis was conducted with MEGA version 2.1 [Kumar et al., 1994]. The evolutionary distance between nucleotide sequences was estimated by the Kimura 2-parameter method.

Nucleotide Sequence Accession Numbers

The GenBank accession numbers for woodchuck nucleotide sequences derived in this study are as follows: AF338270 (wIFNA1a), AF338271 (wIFNA1b), AF425775 (wIFNA1c), AF338272 (wIFNA1d), AF425776 (wIFNA2), AF425777 (wIFNA3), AF338273 (wIFNA4), AF338274 (wIFNA5a), AF425778(wIFNA5b), AF425779 (wIFNA6a), AF425780 (wIFNA6b), AF425781 (wIFNA7), AF338275 (wIFNAP1), AF338276 (wIFNAP2a), AF427140 (wIFNAP2b), and AF427141 (wIFNAP3).

RESULTS

Cloning of Woodchuck IFNA Genes

A cDNA library was constructed from woodchuck PBLs stimulated with pIpC/DEAD dextran and was screened with the human IFNA5 gene as the hybridization probe. Five positive clones were found; after repeated rounds of plaque purification, four independent clones were isolated. Sequencing analysis showed that three of the four positive clones contained the same ORF of 594 nucleotides that codifies for a putative 174 amino acids mature protein with an estimate molecular weight of 20 kDa. This sequence was named wIFNA1a (Fig. 1A). The other clone corresponded to a very similar variant, termed wIFNA1b. To confirm that both sequences existed in the woodchuck and were not generated during either the PCR amplification or sequencing process, or both, these clones were sequenced bidirectionally at least twice, and confirmed by sequencing of at least two independent PCRs reactions from genomic DNA. wIFNA1b showed high homology with wIFNA1a, with a single conservative change at position 460 (A → G) and one nonconservative change into the coding sequence at position 52 (A → G) that lead to a change in amino acid sequence (Ser → Gly at amino acid 18 of the signal peptide). There were two more changes in the noncoding 3′ flanking region: nucleotide +654: A → T and nucleotide +678: G → A (Fig. 1B). At the 3′ end, both sequences share a consensus polyadenylation signal and seven A/U-rich elements (ARE), sequences that alter the stability of the mRNA which is a characteristic of cytokines (Fig. 1A). Analysis of the deduced amino acid sequences showed that both clones codified for proteins with the IFN-α/β/δ family signature: YFHRITVYLKEKKYLPCAW (Fig. 2). Based on structural homology with IFN-α proteins from other species, a hydrophobic signal peptide of 23 aa was determined from the predicted secondary structure (Fig. 2).

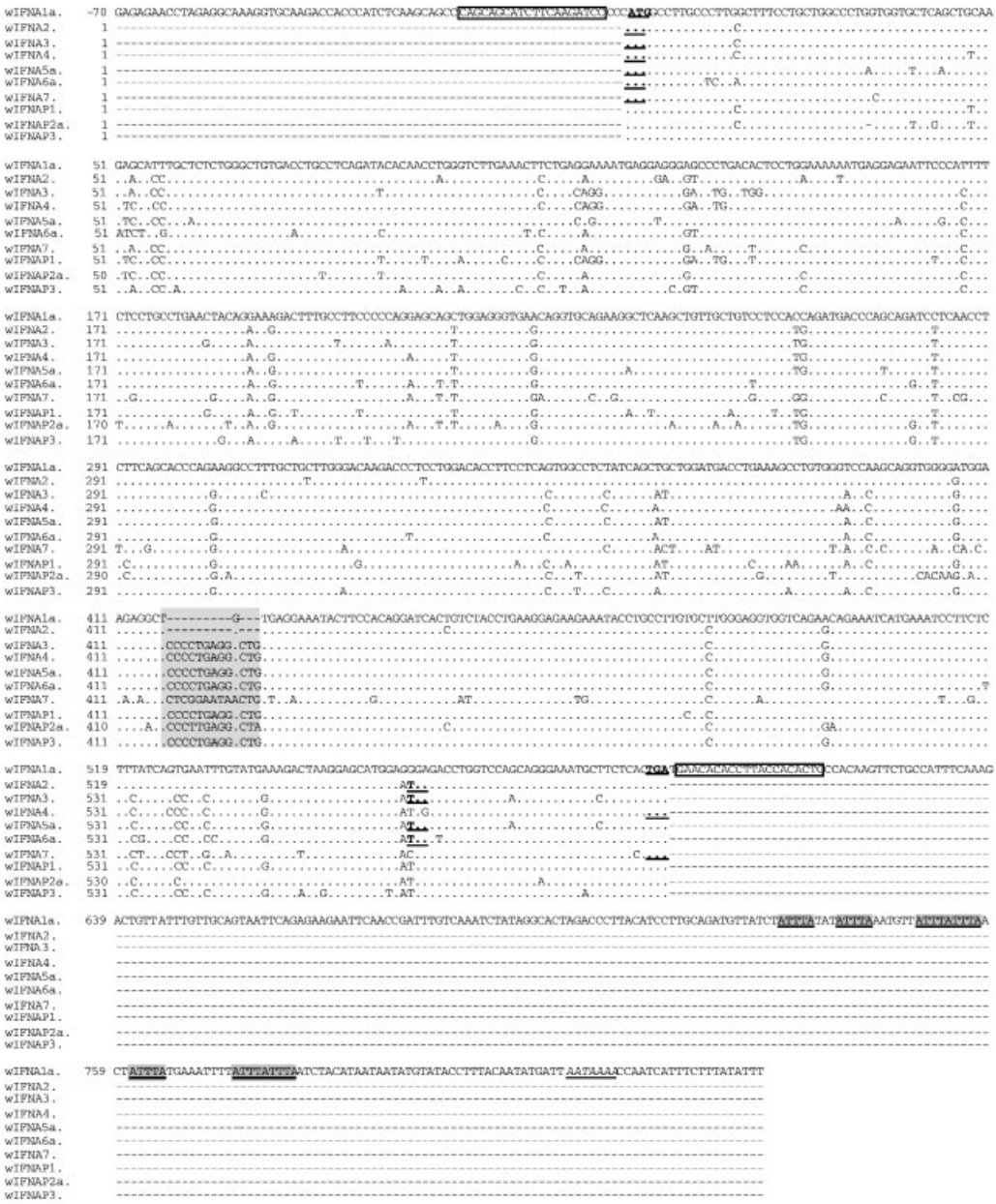
To analyze the woodchuck IFN-α gene family further, a pair of primers were designed according to conserved sequences of the 5′ and 3′ noncoding regions of wIFNA1a and wIFNA1b. Fourteen wIFNA-related sequences were isolated by PCR, using genomic DNA or cDNA as the template. Nucleotide and amino acid sequences are represented in Figures 1 and 2. wIFNA1c and wIFNA1d show a high degree of homology (>99%) to wIFNA1a and wIFNA1b. wIFNA2 codes for the smallest IFN-α protein, a 164 amino acid polypeptide with 95% homology to wIFN-α1 subtypes. wIFNA3, wIFNA5a, wIFNA5b, wIFNA6a, and wIFNA6b code for a deduced mature polypeptide of 167 aa. wIFNA5a and wIFNA5b differ only in a nucleotide at position +276 that leads to a change in one amino acid (His → Gln at amino acid 79). wIFNA6a and wIFNA6b differ in three nucleotides (nucleotide +34: G → C, nucleotide +407: T → A, and nucleotide +534: G → A) that lead to two amino acid change sequences (Val → Leu at amino acid 12 of the signal peptide and Val → Glu at amino acid 153). Sequences wIFNA4 and wIFNA7 code for a polypeptide of 178 aa.

Four of the clones obtained resulted in wIFNA pseudogenes, termed wIFNAP1, wIFNAP2a, wIFNAP2b, and wIFNAP3 (Fig. 1A). In wIFNAP1, the change in nucleotide +244 (C → T) resulted in a stop codon at aa 82. wIFNAP2a and wIFNAP2b present a deletion of a G at nucleotide 33, changing the ORF and presenting a stop codon at aa 60. In wIFNAP3, the change in nucleotide + 112 (G → T) led to a stop codon at aa 28.

The average nucleotide homology among wIFNA sequences is 85%. All wIFN-α are intronless genes. The main differences among all IFN-α were found in the location of the stop codon resulting in proteins of different sizes and a deletion of 12 aa in wIFN-α1 and wIFN-α2 at position +417 (Fig. 1). Comparison of wIFN-α with IFN-α from other species showed an overall identity in nucleotide sequences of approximately 65% and an overall homology in amino acid sequences of 50% (Table I).

To remove nucleotide changes due to DNA polymerase or sequencing errors, a sequence was accepted as wIFNA, if the same sequence was obtained in at least two independent PCRs. A total of eight different PCRs

A)



B)

			Nucleotide									Aminoacid		
		nt no.	52	139	227	406	455	460	480	558	aa no.	S18	24	113
wIFNA1	a		A	T	A	A	T	A	T	G		S	L	M
	b		G	T	A	A	T	G	T	G		G	L	M
	c		A	T	A	G	T	A	C	A		S	L	V
	d		A	G	G	G	C	A	C	A		S	R	V
		nt no.	276								aa no.	79		
wIFNA5	a		T									H		
	b		G									Q		
		nt no.	34	407	534						aa no.	S12	153	
wIFNA6	a		G	T	G							V	V	
	b		C	A	A							L	E	
		nt no.	27											
wIFNP2	a		G											
	b		A											

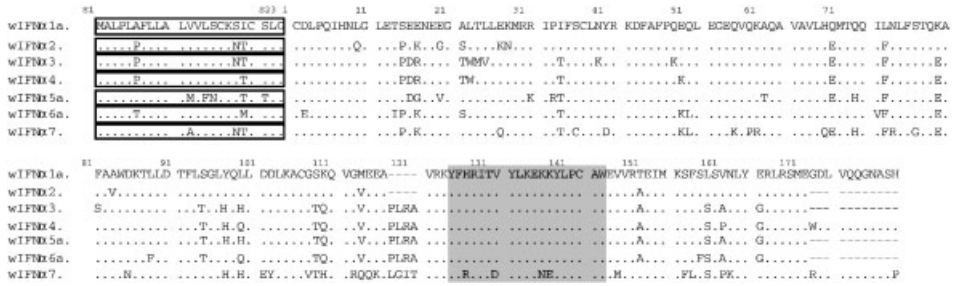


Fig. 2. Alignment of amino acid sequence of woodchuck interferons. Identical amino acids are indicated by dots. Predicted signal peptide are indicated by open boxes. The IFN-α/β/δ family signature is indicated by a shaded box.

were used to characterize all clones, using samples from six woodchucks. Of these eight PCRs, four PCRs were performed with genomic DNA and four with cDNA.

Genomic Analysis of the Woodchuck IFN Gene Family

The number of woodchuck IFN-α genes in the woodchuck genome was estimated by Southern blot analysis (Fig. 3). Woodchuck genomic DNA digested with *Bam*HI resulted in five bands of high molecular weight (15, 12.5, 10.5, 9, and 8 kb), one of which was extremely intense, suggesting that digestion produced a fragment of DNA containing several wIFNA genes.

Eight bands were obtained by digestion with *Hin*dIII (11, 10.5, 9, 8.5, 7.5, 6.2, 5.4, and 3 kb) with three (11, 10.5, and 5.4 kb) of higher intensity, suggesting the presence of more than one interferon gene. Following the criteria that low-intensity bands corresponded to single genes, densitometric analysis of the strongest bands showed that they were just twofold more intense, thus containing two genes. All these data indicate that the woodchuck IFN-α is a multigenic family comprising at least 11 IFN-α subtypes.

Phylogenetic Analysis

The phylogenetic analysis of wIFNA DNA sequences and IFN DNA sequences from different species revealed three different major clusters, corresponding to the subfamilies IFN-α/β/ω. All woodchuck sequences obtained appeared in the IFN-α branch. As described previously for other species, all woodchuck IFNA genes clustered in the same group (Fig. 4).

IFN-α Expression in the Woodchuck Liver

For the analysis of the profile of wIFNA subtypes expressed in the liver, RT-PCR was carried out using primers IFNE1 and IFNE2 from liver samples of three woodchucks. The results shown in Table II indicate that the wIFNA5a gene was expressed most frequently in woodchuck liver constituting 85% of analyzed clones. Only 4 of 27 clones were from a different IFNA subtype (3 wIFNA6a and 1 wIFNA1b). The analysis of woodchuck genomic DNA resulted in a mixture of IFNA subtypes without any predominance, indicating that the result obtained with liver RNA was not influenced by the primer specificity.

Biological Activity of Woodchuck IFN-α

To determine the biological effect of the woodchuck IFN-α subtypes expressed in the liver, their effect was examined on 2–5-OAS expression and on the inhibition of the cytopathic effect induced by a viral infection. To determine the species specificity of woodchuck interferons, their biological activity was compared with that of human IFN-α5, the subtype expressed predominantly in the human liver [Castelruiz et al., 1999].

As shown in Figure 5A, woodchuck cells transfected with plasmids expressing wIFN-α5a and wIFN-α6a showed a 1,000-fold increase in the level of 2–5-OAS mRNA compared with the level of 2–5-OAS mRNA in luciferase-transfected cells. In the case of wIFN-α1b, the increment was 100-fold. Cells transfected with human IFN-α5 showed no significant induction of 2–5-OAS expression.

Figure 5B shows that infection with EMC virus of WCH17 cells incubated with supernatants from mock-transfected cells resulted in a strong cytopathic effect 24 hr after infection. In contrast, WHC17 cells were protected against the destruction by ECM virus infection by the addition of supernatants obtained from cells transfected with woodchuck interferons, being wIFN-α6a antiviral activity slightly stronger than that of wIFN-α5a. Human IFN-α5 showed a weak antiviral activity when tested in WHC17 cells, but proved to be fully active in a standard CPE assay with a human cell line (data not shown).

Taken together, these results indicate that woodchuck IFN-α genes expressed in a eukaryotic expres-

Fig. 1. Alignment of nucleotide sequence of woodchuck interferons. **A:** Alignment of nucleotide sequences of woodchuck interferons. Identical nucleotides are indicated by dots. Putative initiation codon and termination codon of the major open reading frame (ORF) are boldface and underlined. Polyadenylation signal is italicized and underlined. A/U-rich sequences are indicated by a shaded box and underlined. Region of deletion of wIFNA1s and wIFNA2 are indicated by shaded boxes. Primers IFNE1 and IFNE2 are indicated by open box. Sequences wIFNA1b, wIFNA1c, wIFNA1d, wIFNA5b, wIFNA6b, and wIFNAP2b are not shown because of high homology with other sequences. **B:** Changes in nucleotide and amino acid sequences among woodchuck interferons with high homology.

TABLE I. Homology of Open Reading Frame IFN-α of Different Species*

	a)wIFN-A1a	b)wIFN-A2	c)wIFN-A3	d)wIFN-A4	e)wIFN-A5a	f)wIFN-A6a	g)wIFN-A7	h)wIFN-AP1	i)wIFN-AP2a	j)wIFN-AP3	k)hIFN-A5	l)hIFN-A2	m)mIFN-A1	n)rIFN-A1
a)wIFNA1a	—	95.2	90.2	90.9	85.9	85.1	85.9	88.2	88.2	89.7	66.9	67.3	65.3	65
b)wIFNA2	86.3	—	91.7	92.5	91.4	90.9	86.6	89.6	90.2	91.2	67.8	67.9	66.1	66.3
c)wIFNA3	78.7	85.3	—	96.8	95.3	93	87.4	94.7	90.5	93.7	71	70.6	68.5	68.8
d)wIFNA4	90.9	81.6	89.6	—	95.8	94.7	88.6	95.3	92.5	93.8	70	70.3	68.3	69.3
e)wIFNA5a	79.2	82.7	89.5	85.6	—	93.5	87.4	88.1	86.1	92.9	73.7	73.7	70.7	69.6
f)wIFNA6a	80.1	84.8	88.4	86.6	87.4	—	87.1	86.6	85.6	92.4	73	73.8	69.9	68.8
g)wIFNA7	75.7	70.2	70.2	77.7	70.2	70.2	—	85.4	85.9	87.1	64.9	64.2	63.6	64
h)wIFNAP1	—	—	—	—	—	—	—	—	89.7	91.4	83.8	69	68.5	66.9
i)wIFNAP2a	—	—	—	—	—	—	—	—	—	89.7	67.4	66.8	64	63.9
j)wIFNAP3	—	—	—	—	—	—	—	—	—	—	68.6	68.4	67.6	67.8
k)hIFNA5	54.8	58.8	57.2	57.2	58.3	59.3	45.6	—	—	—	—	88.7	74.9	73.9
l)hIFNA2	53.8	57.3	56.2	56.2	56.8	57.8	45.1	—	—	—	82.1	—	76.1	74.8
m)mIFNA1	49	52.2	52.8	52.8	54.8	53.2	46.4	—	—	—	60	61.5	—	89.2
n)rIFNA1	48.5	49	52.8	52.8	51.5	51.5	43.7	—	—	—	58.5	58	80.8	—

wIFN-α, woodchuck interferon.
*The upper right shows identities at nucleotide level; the lower left shows identities at the amino acid level. GenBank accession numbers: NM_002169 (hIFNA5), NM_000605 (hIFNA2), NM_010502 (mIFNA1), X00336 (rIFNA1).

Fig. 3. Southern blot analysis of woodchuck genomic DNA with a human IFN-α5 probe. Woodchuck genomic DNA was subjected to total digestion with the indicated enzymes, electrophoresed through a 0.8% agarose gel, and blotted into a nylon membrane. The membrane was probed with a radiolabeled hIFN-α5 cDNA that included the entire open reading frame (ORF). Arrows mark the DNA hybridizing fragments obtained after digestion with *Hin*d III. The molecular weight was estimated by running in a parallel cane 4 μl of DNA molecular weight marker X (Bommainger 6MBM, Mannheim, Germany).

sion system are functional, exhibiting potent antiviral activity. They also reflect a high degree of species specificity.

DISCUSSION

The IFN-α system is a family of related proteins coded from distinct genes, in which a high incidence of allelic variations has been detected [Velan et al., 1985]. Multiple IFNA genes have been detected in every mammalian species in which they have been sought. In this study, the woodchuck IFN-α family was cloned and analyzed as a first step toward its therapeutic application in this useful animal model of hepatitis B virus (HBV) infection. It was found that the woodchuck IFN-α proteins are coded by a multigenic family composed of at least 10 subtypes. By comparison, the human IFN-α family has 14 subtypes and there are more than 10 in the mouse [De Mayer and De Mayer-Guignard, 1998]. The reason for this redundancy remains unknown. However, it seems plausible that the different IFN-α subtypes, although sharing some activities, have different functional profiles and different transcriptional activation in response to external stimuli. Superfluous members would be either eliminated rapidly or

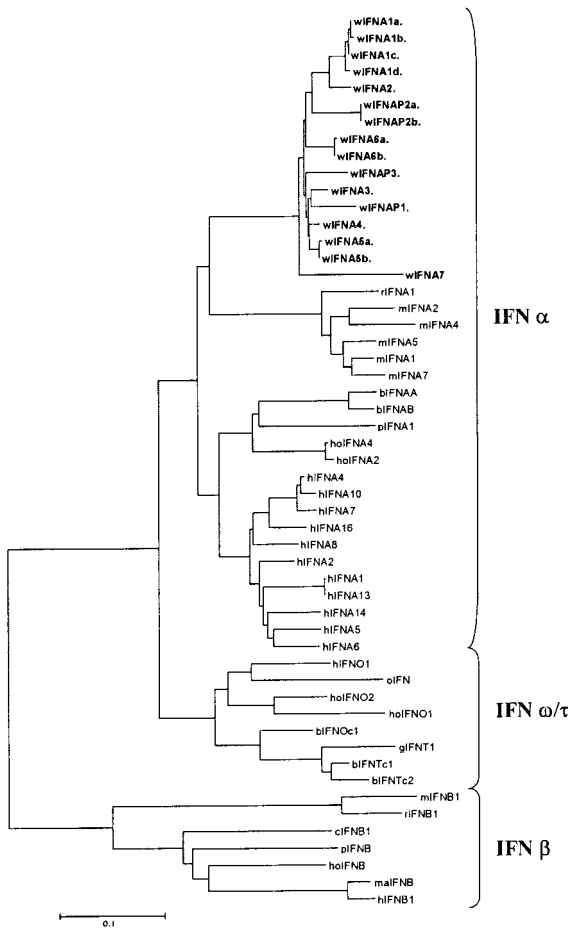


Fig. 4. Phylogenetic tree based on nucleotide sequence of woodchuck interferon-α and type I IFN from different species. Woodchuck interferons are boldface. GenBank accession numbers of sequences used in the tree: X00336 (rIFNA1), NM_010502 (mIFNA1), NM_010503 (mIFNA2), NM_010504 (mIFNA4), NM_010505 (mIFNA5), NM_008334 (mIFNA7), NM_010510 (mIFNB1), NM_019127 (rIFNB1), M10952 (bIFNAA), M10953 (bIFNAB), AF238613 (bIFNOc1), AF339094 (bIFNTc1), AF196323 (bIFNTc2), X57191 (pIFNA1), S41178 (pIFNB), A33687 (hoIFNA2), A33695 (hoIFNA4), A33689 (hoIFNO1), A33687 (hoIFNO2), A33685 (hoIFNB), NM_024013 (hIFNA1), NM_000605 (hIFNA5), NM_021068 (hIFNA4), NM_002169 (hIFNA5), NM_021002 (hIFNA6), NM_021057 (hIFNA7), NM_002170 (hIFNA8), NM_002171 (hIFNA10), NM_006900 (hIFNA13), NM_002172 (hIFNA14), NM_002173 (hIFNA16), NM_002177 (hIFNO1), NM_002176 (hIFNB1), AJ011909 (maIFNB), U26254 (oIFN), U55050 (gIFNT1), and Ab021707 (cIFNB).

converted to pseudogenes unless they conferred some selective advantage [Hughes, 1994].

Screening a woodchuck cDNA library first and genomic DNA afterward permitted the identification of 16 wIFNA sequences tentatively ascribed to 7 functional subtypes and 5 allelic variations and 4 pseudogenes. With the available data, it is not possible to determine with certainty which sequences are allelic variants of a subtype and which are different subtypes. Seven has been proposed as the number, according to data from the phylogenetic analysis of woodchuck interferons. All wIFNA genes are intronless. The analysis of deduced amino acid sequences shows a signal sequence polypeptide with 23 amino acids common to all IFN-α proteins described. As described in human IFN-α, wIFN-α have four cysteines at similar positions which could be involved in the formation of disulfide bridges (amino acids 1–106, 36–140), suggesting a common tertiary structure.

TABLE II. Expression of Subtypes of wIFN in the Liver

Woodchuck	wIFNA5a	wIFNA6a	wIFNA1b
A	66% (6/9)	22% (2/9)	11% (1/9)
B	100% (10/10)	—	—
C	87.5% (7/8)	12.5% (1/8)	—
Total	85% (23/27)	11% (3/27)	3.7% (1/27)

wIFN, woodchuck interferon.

The main differences observed among the wIFN-α products is the length of the proteins. This feature has been reported for other species, such as the mouse, pig, and horse, but not for human IFN-α , for which different subtypes have the same size. This size variation, where described, has been related to deletions in the nucleotide sequence. This is the case of wIFNA1 and wIFNA2 subtypes, which present, in comparison with the other wIFNA sequences, a deletion of 12 nucleotides at position +417. However, wIFNA have another source of size variation that is the location of the stop codon. Two possible sites for the stop codon have been described, with a mutational event present in the longest sequences inactivating the first stop codon. A similar situation has been described in the 6-amino acid tail of IFN-τ and IFN-ω [Roberts et al., 1998].

Phylogenetic analysis shows that all wIFNA sequences form a group inside the branch of IFN-α. The wIFNA cluster with interferons from other rodents, such as rat and mouse interferons, originating an outgroup with respect to the rest of mammalian species. Our results support further data suggesting than rodents IFNA genes were among the earliest of the eutherian order to diverge [Hughes, 1995].

Investigation of the wIFNA subtypes expressed in woodchuck liver has shown that wIFNA5a is the main subtype present in woodchuck liver (85%). This situation is similar to what is found in humans, where hIFNA5 is the predominant IFNA subtype expressed in the liver [Castelruiz et al., 1999]. The abundance of a specific subtype in this organ might suggest tissue-specific functions for this particular subtype.

The functional analysis of the three wIFNA subtypes expressed in the liver demonstrated potent antiviral activity of wIFN-α5a, wIFN-α6a, and wIFN-α1b, as well as a strong induction of a typical interferon inducible gene, the antiviral protein 2–5-OAS. One of the characteristics of IFN-α is species specificity. This is confirmed by the present study, showing a very weak antiviral effect of human IFN-α5 on a woodchuck cell line. The results stress the need of using species-specific IFNA sequence to explore the biological and antiviral properties of IFN-α in experimental animals. Thus, the cloning and characterization of the woodchuck IFN-α family paves the way for systematic studies of the biological and therapeutic effects of this

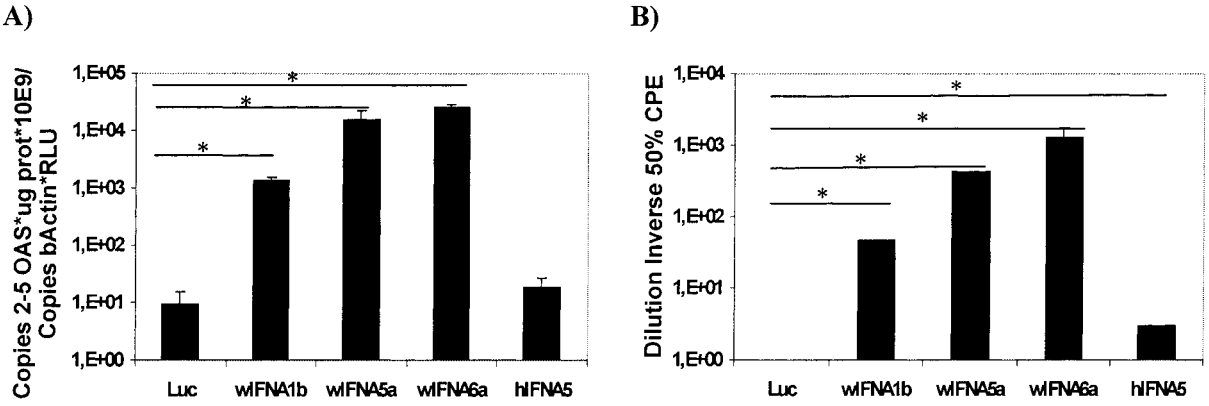


Fig. 5. Biological activity of woodchuck interferons and human IFN-α5 in woodchuck hepatic cell line (WCH17). **A:** Expression of 2–5-oligoadenylate synthetase (2–5-OAS) in woodchuck cells transfected with plasmid expressing the IFN-α genes analyzed as described in Materials and Methods. **B:** Inhibition of the cytopathic effect induced by encephalomyocarditis (EMC) virus on WHC17 cells after incubation with IFN-α, analyzed and expressed as described in Materials and Methods. Mean value and standard deviation of triplicates are shown. $^*P < 0.05$.

cytokine or combination therapies and of the efficacy of different ways of administration of interferons. It also provides a useful tool for the study of the antiviral mechanisms mediated by IFN-α in vivo.

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REFERENCES

Aurisicchio L, Delmastro P, Salucci V, Paz OG, Rovere P, Ciliberto G, La Monica N, Palombo F. 2000. Liver-specific alpha 2 interferon gene expression results in protection from induced hepatitis. J Virol 74:4816–4823.

Biron CA. 2001. Interferons alpha and beta as immune regulators—a new look. Immunity 14:661–664.

Castelruiz Y, Larrea E, Boya P, Civeira MP, Prieto J. 1999. Interferon alpha subtypes and levels of type I interferons in the liver and peripheral mononuclear cells in patients with chronic hepatitis C and controls. Hepatology 29:1900–1904.

David M. 1995. Transcription factors in interferon signaling. Pharmacol Ther 65:149–161.

De Mayer E, De Mayer-Guignard J. 1991. Interferons. In: Thompson A., editor. The cytoquinehandbook. London: Academic Press. p 215–239.

Der SD, Zhou A, Williams BR, Silverman RH. 1998. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc Natl Acad Sci USA 95:15623–15628.

Feitelson MA, Marion PL, Robinson WS. 1981. Antigenic and structural relationships of the surface antigens of hepatitis B virus, ground squirrel hepatitis virus, and woodchuck hepatitis virus. J Virol 39:447–454.

Fleischmann WR, Masoor J, Wu TY, Fleischmann CM. 1998. Orally administered IFN-alpha acts alone and in synergistic combination with intraperitoneally administered IFN-gamma to exert an antitumor effect against B16 melanoma in mice. J Interferon Cytokine Res 18:17–20.

Galibert F, Chen TN, Mandart E. 1982. Nucleotide sequence of a cloned woodchuck hepatitis virus genome: comparison with the hepatitis B virus sequence. J Virol 41:51–65.

Hervas-Stubbs S, Lasarte JJ, Sarobe P, Prieto J, Cullen J, Roggendorf M, Borras-Cuesta F. 1997. Therapeutic vaccination of woodchucks against chronic woodchuck hepatitis virus infection. J Hepatol 27:726–737.

Hughes AL. 1994. The evolution of functionally novel proteins after gene duplication. Proc R Soc Lond B Biol Sci 256:119–124.

Hughes AL. 1995. The evolution of the type I interferon gene family in mammals. J Mol Evol 41:539–548.

Issacs D, Lindenman J. 1957. Virus interference. 1. The interferon. Proc R Soc Lond B 147:258–273.

Kaplan H, Carandang G, Mandigo G, Newman R, Yousefi S, Chiu J, Tilles JG, Cesario TC. 1987. Optimal conditions for the induction of human mononuclear leukocytes by complexed polyinosinic-polycytidylic acid. J Clin Lab Immunol 22:153–156.

Kumar S, Tamura K, Nei M. 1994. MEGA: Molecular evolutionary genetics analysis software for microcomputers. Comput Appl Biosci 10:189–191.

Maddrey WC. 2000. Hepatitis B: an important public health issue. J Med Virol 61:362–366.

Malaguarnera M, Restuccia S, Ferlito L, Mazzoleni G, Giugno I, Pistone G. 2001. Antiviral drugs in chronic hepatitis B: review and meta-analysis. Int J Clin Pharmacol Ther 39:4–11.

Menne S, Tennant BC. 1999. Unraveling hepatitis B virus infection of mice and men (and woodchucks and ducks). Nat Med 5:1125–1126.

Protzer U, Nassal M, Chiang PW, Kirschfink M, Schaller H. 1999. Interferon gene transfer by a hepatitis B virus vector efficiently suppresses wild-type virus infection. Proc Natl Acad Sci USA 96:10818–10823.

Roberts RM, Liu L, Guo Q, Leaman D, Bixby J. 1998. The evolution of the type I interferons. J Interferon Cytokine Res 18:805–816.

Roggendorf M, Tolle TK. 1995. The woodchuck: an animal model for hepatitis B virus infection in man. Intervirology 38:100–112.

Velan B, Cohen S, Grosfeld H, Leitner M, Shafferman A. 1985. Bovine interferon alpha genes. Structure and expression. J Biol Chem 260:5498–5504.

Yamagata Y, Yuasa Y, Yamamoto K, Okamoto K, Igari Y, Iga K, Ogawa Y. 2000. Pharmacologic effect of recombinant human IFN-alpha, continuously released from a matrix prepared from a polyglycerol ester of fatty acids, on 2′,5′-oligoadenylate synthetase activity in murine liver. J Interferon Cytokine Res 20:153–160.

Zeuzem S, Feinman SV, Rasenack J, Heathcote EJ, Lai MY, Gane E, O'Grady J, Reichen J, Diago M, Lin A, Hoffman J, Brunda MJ. 2000. Peginterferon alpha-2a in patients with chronic hepatitis C. N Engl J Med 343:1666–1672.