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GENE STRUCTURE OF HUMAN INDOLEAMINE 2,3-DIOXYGENASE[1]

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Summary: Two genomic DNA clones that encode human indoleamine 2,3-dioxygenase (IDO) were isolated from the human genomic DNA library using the IDO cDNA as a probe, and their restriction maps and partial nucleotide sequences were determined. The human IDO gene spanned 15 kilobase pairs with ten exons. The 5' terminus of the IDO mRNA was 33 nucleotides upstream of the translation initiation codon ATG. The 5' flanking region contained ISRE, X-box, and Y-box like sequences. Southern blot analysis of the human genomic DNA indicated that the human IDO gene was present in a single copy in the genome.

Interferons (IFNs) are a family of cytokines with antiproliferative, antiviral, and immunomodulatory properties [1,2]. All IFNs stimulate to induce cellular RNAs and some new proteins. A large number of cDNAs corresponding to IFN-induced mRNAs have been isolated and their sequences determined. In recent years the biological activities of IFN-induced proteins have been identified and their roles have been established [3].

It has been reported that the activities of some enzymes are elevated by IFNs, such as the 2', 5'-oligoadenylate synthetase, the P1 kinase, the indoleamine 2, 3-dioxygenase, the GTP cyclohydrolase I and the tryptophanyl-tRNA synthetase [3-6]. One of them, that is the indoleamine 2,3-dioxygenase(IDO), is a monomeric hemoprotein that catalyzes the oxidation of an essential amino acid, tryptophan, into N-formyl kynurenine in the first step of the kynurenine pathway and is widely distributed in extrahepatic tissues such as the placenta, the lungs and the small intestine of humans and other mammals [7,8]. IDO is induced by interferon-γ (IFN-γ) dramatically (up to 100-fold) in many types of human cell lines in vitro, resulting in the depletion of tryptophan in the culture medium and cell growth inhibition [9]. Furthermore, IDO was induced by IFN-γ in allogenic or synergic tumor cells undergoing rejection [10]. With a similar mechanism, IDO which was induced by IFN-γ blocked the

[1] Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. M86472 - M86481.

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Abbreviations: IDO, indoleamine 2, 3-dioxygenase; IFN, interferon; kb, kilobase pairs; bp, base pairs; PCR, polymerase chain reactions; ISRE, interferon-stimulable response element; MHC, major histocompatibility complex; IRF-1, interferon regulatory factor -1.

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replication of intracellular parasites [11]. In these studies, it has been suggested that tryptophan depletion by IFN-γ induced IDO may be one of the causes of the antiproliferative effect and the antitumor activity of IFN-γ.

We have previously isolated the human IDO cDNA [12] and reported that IFN-γ mediated induction of IDO occurs at a level of RNA transcription. A major 1.7 kilobase pairs (kb) and a minor 2.3 kb mRNA were detected by Northern blot analysis using IDO cDNA as a probe in IFN-γ treated cells. Furthermore, we found that the 2.3 kb mRNA had an additional 0.6 kb non-coding sequence in its 5'end (S. Tone, *et al. in preparation*). In an attempt to analyze the structure of the human IDO gene, we isolated the full length human IDO gene that corresponded to the cDNA and determined the exon-intron organization, the total number in the genome and the sequence of the 5'-flanking region.

MATERIALS AND METHODS

Interferon

Pure recombinant human IFN-γ with specific activities of 6.0×10^6 units/mg was generously provided by Dr. M. Okabe, Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan.

Cells and growth conditions

A431 cells were obtained from the Japanese Cancer Research Resources Bank (JCRB). The culture medium was Dulbecco's modified Eagle's MEM and 10% fetal calf serum (Bocknek Laboratories Inc., Ontario, Canada). A431 cells were cultured at 37°C in humidified air at 5% CO_2.

Screening of a genomic library and restriction enzyme mapping

A human genomic DNA library from peripheral blood leukocytes of a Japanese person, containing BamH I partial digests in λEMBL 3, was obtained from Dr. Y. Sakaki, Research Laboratory for Genetic Information, Kyushu University through JCRB. The library was screened using ^{32}P-labeled human IDO cDNA [12] as a probe. Two overlapping clones, HIDO 14 and HIDO 15, were isolated and mapped with restriction enzymes.

Nucleotide sequence analysis

Restriction fragments were subcloned into pBluescript/KS+ and sequenced by the dideoxy chain termination method [13]. Oligonucleotide primers for the exons and the introns were synthesized to determine the sequence of exon-intron junctions. These primers were also used to determine the localization of the exons and the lengths of the introns using polymerase chain reactions (PCR) [14].

Southern blot analysis of human genomic DNA

Human placenta genomic DNA (10 μg) was digested with suitable restriction enzymes, electrophoresed in a 0.7% agarose gel, transferred onto a nitrocellulose membrane and hybridized to a labeled human IDO cDNA.

S1 nuclease mapping of the 5' terminus of RNA

To determine the 5' end of the mRNA according to the procedure previously reported [15], a primer (5' GTGTGCCATTCTTGTAGTCT 3'), complementary to position 12 to 31 of IDO cDNA, was 5' end labeled with [γ-^{32}P] ATP and hybridized to a 520 base pairs (bp) Stu I-Hinc II fragment containing the promoter-exon 1 of IDO gene inserted into pBluescript/KS+ (Fig. 1, 4). This hybrid was extended by using Klenow fragment and digested with Sac I to give the probe a defined 3' end. This probe was hybridized with each 20 μg total RNA from IFN-γ (1000 U/ml) treated/untreated A431 cells, or liver as a negative control. After digestion with S1 nuclease, the products were subjected to 8% polyacrylamide urea gel electrophoresis. For the size marker, single-stranded phage M13mp18 was sequenced with the -40 primer by using the dideoxy chain termination method.

RESULTS AND DISCUSSION

We isolated ten overlapping positive clones from λEMBL3 recombinant phages of a human genomic library (10^6 p.f.u.) from peripheral blood leukocytes of a Japanese person using a probe derived from human IDO cDNA [12]. Two of these clones, HIDO 14 and HIDO 15, were found to contain the whole IDO gene by using gene mapping analysis (Fig. 1). The human IDO gene spanned 15 kilobase pairs with ten exons and nine introns. The restriction map of the human IDO gene was shown in Fig. 1. The nucleotide sequence around exon-intron junctions was determined and exon-intron organization was revealed (Table 1). Comparison of the DNA sequence data with that of the cDNA which we have reported showed that the cDNA sequence was composed of ten exons and the nucleotide sequences of the exons were identical to those of the cDNA. The size of the introns was confirmed by the restriction mapping and polymerase chain reactions using exon specific primers. The RNA splice junctions were homologous to the consensus sequences except for intron 7. At the 5' end of intron 7 the dinucleotide "gc" was found instead of the consensus dinucleotide "gt" [16]. It is possible that the gene containing this rare non-conforming splice site may function as a crucial node in the regulation of the genetic pathway of cell growth and differentiation [16].

In order to determine the total number of IDO genes in the genome, Southern blot analysis of human genomic DNA was performed, using the human IDO cDNA as the probe (Fig. 2). The pattern of hybridization bands was consistent with the restriction map of the cloned IDO gene, suggesting that the human IDO gene was present in a single copy per haploid genome (Fig. 1 and 2).

To define the potential transcription start site, S1 nuclease mapping was performed. It revealed three major and a few minor transcription start sites (Fig. 3). Measurement of the S1 product size allowed us to localize the putative transcription start sites. We found that TATA-like sequences were present in the upstream regions of each initiation site and also CCAAT box

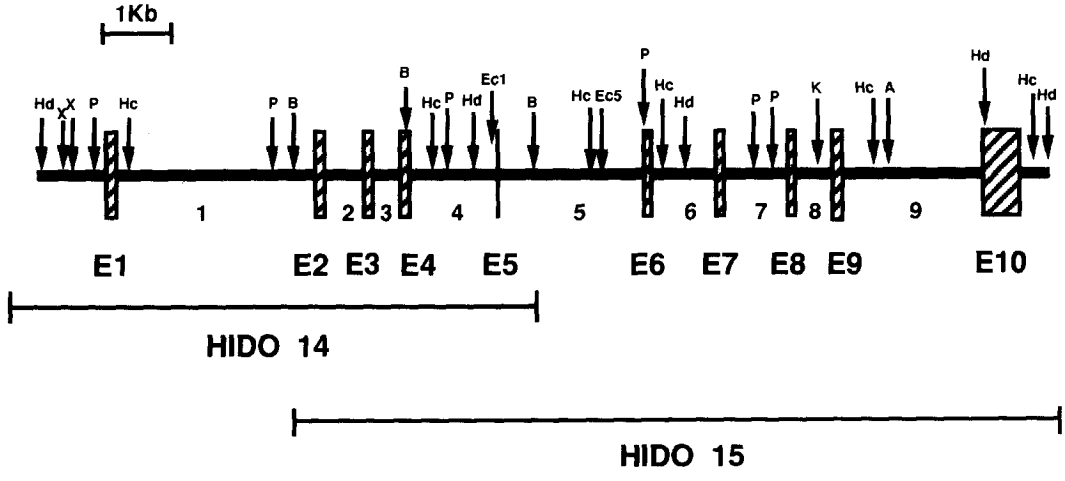

Fig. 1. Restriction endonuclease map of the human IDO gene. The genomic clones HIDO 14 and HIDO 15 are depicted as bars. Exons are represented by hatched boxes. The following restriction sites are indicated: A, Acc I; B, BamH I; Ec1, EcoR I; Ec5, EcoR V; Hc, Hinc II; Hd, Hind III; K, Kpn I; P, Pst I; X, Xba I.

Table 1. Exon/Intron boundaries and size of each intron in the human IDO gene[a]

Exon[b]	Length	5'splice[c]	Intron[b]	Length[d]	3'splice[c]
	bp			kb	
E1	120	CACAGgtaaga	1	3.2	ttttttttcaagGAA
E2	96	AGAAGgtttga	2	0.9	ccatttgttttcagTTA
E3	120	GTAAGgtttgg	3	0.5	tgtattttaatcagGTC
E4	119	AATAAgtatgt	4	1.4	gctgctttcataagGCC
E5	15	TATGAgtaagt	5	2.3	tccaatttcctcagGAA
E6	100	TCAAAgtacgt	6	1.2	tttgtttgttttagGTA
E7	118	CCACGgcaagt	7	1.2	ccatctttttacagATC
E8	52	TCTGGgtatgt	8	0.5	ctttcctctgatagCTG
E9	149	TGGAGgtgagt	9	2.4	ctcttttcctatagGAC
E10	596				

[a]Exon sequences are in capital letters; intron sequences are in small letters. The underlined sequences are not consistent with the consensus dinucleotides of 5' splice site.
[b]Numbered as in Fig. 1.
[c]The consensus 5' splice sequences are AGgta/gagt and the consensus 3' splice sequences are t/ct/cttt/ct/ct/ct/ct/ct/cncagG [16].
[d]Intron length estimated by PCR.

like sequences were present in further upstream regions from these TATA boxes (Fig. 4). From these data we deduced that the 5' terminus of the 1.7 kb mRNA for the human IDO was 33 nucleotides upstream of the translation initiation codon ATG (Fig. 4). The transcription start sites were also confirmed by a primer extension experiment (data not shown). In addition, the sequence from nucleotide -481 to -475 was a potential binding site for AP-1, which had been known as the *ras*- and phorbol ester-responsive enhancer element [17,18] (Fig. 4).

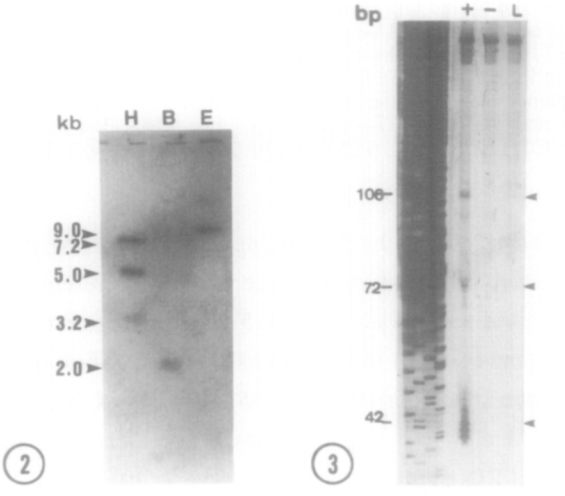

Fig. 2. Southern blot analysis of genomic DNA. Human genomic DNA from a placenta was digested with BamH I (lane B), EcoR I (lane E), and Hind III (lane H). Hybridization was carried out with a ^{32}P- labeled human IDO cDNA as a probe. The size markers are indicated in kilobase pairs (kb).

Fig. 3. Determination of the 5' termini of the human IDO mRNAs by S1 mapping. The probe was hybridized to total RNA from A431 treated (lane +) or untreated (lane -) with IFN-γ (1000 u/ml) and liver (lane L) as a negative control. The bp denotes base pairs. The arrowheads indicate the 5' end of the individual S1-protected products.

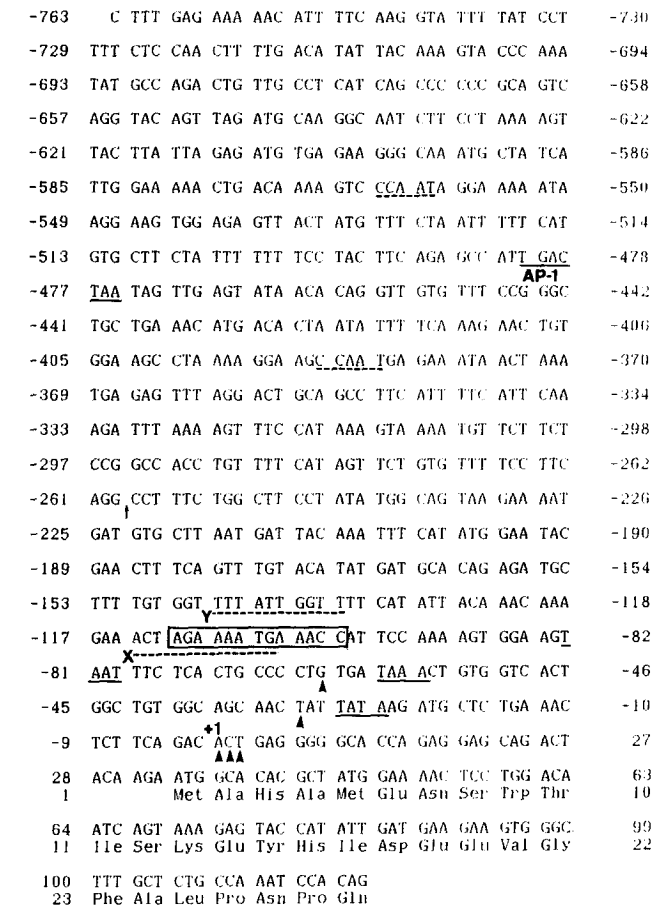

Fig. 4. Exon 1 and the 5'-flanking region of the human IDO gene. Amino acids are given in three-letter codes below the DNA sequence. Major transcription start sites are indicated by arrowheads. "+1" is 33 nucleotides upstream of the translation initiation codon ATG. The arrow denotes Stu I site. TATAbox like sequences and the AP-1 site are underlined. X,Ybox and CCAAT sequences are dot-underlined. The box shows the ISRE sequence.
Consensus sequence: AP-1, 5'-TGANTA/CA-3' [17]; ISRE, 5'-GGAAAN(N)GAAACT-3' [19]; X-box, 5'-CCC/TAGA/CA/GACNG-3' [20, 21]; Y-box, 5'-CTGATTGGT/CT/C-3' [20, 21].

Further sequence analysis of the 5'-flanking region also revealed that the conserved region contained an interferon-responsive sequence which was present in other IFN-stimulated genes, and was the target of the IFN action. The 5'-flanking region contained the interferon-stimulable response element (ISRE) (nucleotides -111/-99), X-box (nucleotides -114/-104), and Y-box like sequence (nucleotides -144/-135) (Fig. 4). X-box and ISRE overlapped between nucleotides -111 and -104. ISRE is known to match a common sequence that is present in many different IFN-stimulated gene promoters [19]. X and Y boxes are essential components of the IFN-γ response region of major histocompatibility complex (MHC) class II promoters [20, 21]. Their motifs occurred in an inverted orientation in the 5'-flanking region of the human IDO gene (Fig. 4). There is also a similar case, where a second pair, referred to as X'-Y', was found in reverse orientation in the upstream control region of MHC class II Eα, Aβ and Eβ [21]. From these sequence analysises, it would appear that the 5'-flanking region of the human IDO gene contains responsive elements to IFN-γ. Beside these elements, there were six

sequences which were normally or complementarily identical to the interferon regulatory factor-1 (IRF-1) binding sequence within this region (not shown) [22]. The IRF-1 binding sequence is the functional repeated hexanucleotide sequence, and the IRF-1 specifically binds to these cis-elements within the promoter of IFN-β and some IFN-inducible genes [22].

We have already found that two mRNAs (the 2.3 kb and the 1.7 kb mRNA) of IDO were transcribed from a single gene (S.Tone, *et al. in preparation*). Recently, Dai and Gupta have identified the transcription start site for the 2.3 kb mRNA of the human IDO and have reported that the ISRE and X-box like sequence with enhancer activity for mRNA of the IDO gene resided upstream from the transcription start site of the 2.3 kb mRNA [23]. It would be likely that the transcription of the 1.7 kb mRNA and the 2.3 kb mRNA of IDO would be controlled by single upstream regulatory element, or primarily the 2.3 kb mRNA would be transcribed from the IDO gene and subsequently the 1.7 kb mRNA would be produced from the 2.3 kb mRNA by RNA processing. Further analysis by chloramphenicol acetyl transferase assay and DNA foot printing will serve to examine these elements in the 5'-flanking region to be functional in the regulation of the IDO gene expression.

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