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Nucleotide sequence of a cDNA encoding human tumor necrosis factor β from B lymphoblastoid cell RPMI 1788

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Published sequences of cDNA for human tumor necrosis factor β (TNF-β) have a discrepancy within the coding region as well as exon 1. To resolve these discrepancies we have re-isolated TNF-β cDNA from the human B cell lymphoblastoid cell line, RPMI 1788, and determined its DNA sequence. Results indicate that amino acid 26 is threonine (Thr) instead of asparagine (Asn). In contrast to published sequences, the sequence of the exon 1 region corresponded to the genomic sequence of TNF-β. From our studies we conclude that the TNF-β gene of the human B cell lymphoblastoid cell line, RPMI 1788, is homologous with respect to the TNF-β gene.

cDNA cloning; Tumor necrosis factor β; RPMI 1788 cell; Polymerase chain reaction

1. INTRODUCTION

Human tumor necrosis factor β (TNF-β; or lymphotoxin LT) is a lymphokine secreted from mitogen-activated peripheral blood leukocytes [1] and cell lines of hematopoietic origin [2]. It is highly cytotoxic to a wide range of tumor cells in vitro and in vivo [3,4] and has been reported to be heterogenous with regard to size and charge [5]. TNF-β derived from the B lymphoblastoid RPMI 1788 cell line has been isolated and its amino acid sequence determined [6].

The molecular cloning of cDNA for TNF-β from peripheral blood mononuclear cells (PMBC) [4] and the genomic sequence of this gene has already been reported [7,8], whilst Kobayashi et al. [9] isolated cDNA from the human T cell hybridoma cell line, AC5-8, which had been stimulated with phorbol myristate acetate (PMA) and concanavalin A (Con A). There is a discrepancy between these two studies with respect to the DNA sequences corresponding to amino acid (aa) 26; Kobayashi et al. [9] and Nedwin et al. [7] reported aa 26 to be Asn, while others [4,8] claimed that it was Thr. The confusion in the literature was not limited to the coding region. A part of the sequence of the exon 1 region, which had been determined from the cDNA [4,9], was not found in the genomic sequence [7,8] of the same gene.

In order to settle these discrepancies and consequent confusion, we have isolated the TNF-β cDNA gene and determined the coding region as well as the exon 1 sequence. In addition we have also isolated the TNF-β gene fragment from genomic DNA by the polymerase chain reaction (PCR) and found that aa 26 is Thr and the exon 1 region of the cDNA does indeed have the sequence corresponding to the genomic sequence.

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2. MATERIALS AND METHODS

2.1. *Materials*

Restriction enzymes and DNA modifying enzymes were obtained from Amersham, Takara Shuzo, Boehringer-Mannheim, New England Biolabs and Toyobo. The cDNA synthesis kit was purchased from Amersham. DNA sequencing reagents were obtained from the US Biochemical Corp. λgt11 was purchased from Promega.

2.2. *Cells*

RPMI 1788 cells were purchased from ATCC (#CCL156) and were maintained in RPMI 1640 (Gibco) medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 100 U/ml of penicillin and 100 μg/ml of streptomycin. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.3. *RNA isolation and construction of a cDNA library*

Total RNA (4.9 mg) was extracted from 1×10^9 cells of RPMI 1788 cells by the guanidine isothiocyanate–CsCl method [10]. Poly(A)$^+$ RNA (115 μg) was purified from total RNA (2.2 mg) by an oligo(dT)cellulose column [11]. Double-stranded cDNA (10 μg) was synthesized by the method of Gubler and Hoffman [12]. The ends were filled in with T4 polymerase and Klenow fragments and the cDNA was treated with *Eco*RI methylase (New England Biolabs.). Phosphorylated *Eco*RI linkers were added [10]. cDNA fragments greater than approximately 600 bp were collected by low melting point agarose (LMA) (Bio-Rad) and were ligated into the *Eco*RI site of λgt11 phage vector [13]. In vitro packaging (Stratagene) produced a library of 2.5×10^5 independent plaques.

2.4. *Isolation of TNF-β cDNA clones*

The oligonucleotide probes were synthesized with the DNA synthesizer (Applied Biosystems; ABI): 5′-ACC AGG GAG CCC CTG

GGC CCC AGG CAG CAG-3' (S1) corresponding to the amino acid residues −7 to 3 of TNF-β and 5'-GGA GGA GGT GGC CTT GGG AGA GTA GGC TTT CCC AGA-3' (S2) corresponding to residues 82–93 of TNF-β. The recombinant phage was plated on a 15 cm plate and screened by hybridizing with ^{32}P-labeled probes in 6× SCC, 5× Denhardt's solution, salmon testis DNA (100 μg/ml), and 0.5% SDS at 65°C for 16 h. Filters were washed twice in 6× SSC at room temperature for 15 min then washed twice in 6× SSC at 65°C for 15 min. Phage DNA was purified after plaque purification of positive clones [10], and was subcloned into pTZ19U or pTZ18U [14]. Both strands of the inserts were sequenced with modified T7 polymerase (US Biochemicals) and analyzed with an ABI DNA sequencer. Ambiguous regions of sequence were confirmed by subcloning fragments into pTZ19U or pTZ18U followed by the dideoxy sequencing method [15].

2.5. Cloning of the genomic DNA

Genomic DNA was isolated from RPMI 1788 cells as described by Maniatis et al. [10] except that 1% SDS was used instead of 0.5%, and proteinase K was added to a final concentration of 1 mg/ml and RNAse T1 to 7.1 μg/ml. Genomic DNA (660 ng, approximately 2.2×10^5 molecules) was subjected to 35 cycles of amplification [16] in a Tempcycles Model 50 (Coy Laboratory Products Inc.) with primer 5'-GAA TTC CAC CTG GGA GTA GAC GAA GTA-3' (P1), which represents the bases 1927–1947 of the genomic DNA of TNF-β [7], and 5'-CCA TCT CCT TGG GCT GCC CG-3' (P2) which corresponds to the bases 852–871 of the genomic DNA of TNF-β [7] (see Fig. 1). The reaction mixture contained 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 0.01% (w/v) gelatin, 200 μM dNTPs, 1 μM of each primer, 2.5 U *Taq* polymerase (Takara Shuzo), and 1 U of the Perfect Match Enhancer (Stratagene) in a final volume of 100 μl. The mixtures were covered with 2 drops of mineral oil (Sigma). Thermal cycling conditions were 1 min at 95°C, 2 min at 60°C and 3 min at 72°C. Amplification was completed by a final incubation at 72°C for 10 min. The PCR products were subjected to 0.8% agarose gel electrophoresis for analysis and purified by LMA and ligated with pTZ19U or pTZ18U. After transformation of *Escherichia coli* strain NM522 [17] with these plasmids, white colonies from X-gal/IPTG plates were screened and the DNA sequences were determined.

3. RESULTS AND DISCUSSION

We screened 5×10^4 clones from the RPMI 1788 cDNA library with two synthetic oligonucleotide probes (S1 and S2) and isolated two positive clones (LT#2 and LT#11). As shown in Fig. 1, the sequence consists of 615 bp (81–695) of an open reading frame, which gives the amino acid sequence of the TNF-β protein sequence reported by Aggarwal et al. [6], 139 bp (60–80) of 5' untranslated sequence, 627 bp (699–1325) of 3' untranslated sequence, and the consensus polyadenylation additional signal [18] (1307–1312) just upstream of the poly(A) tail. The 26th amino acid turned out to be threonine in contrast to the reports by Kobayashi et al. [9]. This TNF-β cDNA was inserted into the mammalian expression vector, pcDL-SRα296. COS-1 cells containing this vector excreted cytotoxic factor and showed cytotoxic activity against L929 cells (data not shown).

To confirm that our cDNA sequence obtained from RPMI 1788 is in accordance with the genomic DNA, PCR was performed with the genomic DNA as a template using the primers, P1 and P2 (Fig. 1). The PCR product was subcloned and sequenced and it was found that the sequence obtained from the genome was identical to that of the cDNA.

The comparison of cDNA and genomic DNA of TNF-β, which has been published, and our results are shown in Table I. First, our TNF-β cDNA corresponding to the exon 1 region was identified with our genomic DNA sequence from the same source. Dotted cDNA sequences of Gray's [4] and Kobayashi's [9], as shown in Table I, were not found in our and others' genomic sequences [7,8,19,20]. It is unlikely that these sequences of the exon 1 region came from different splicing of messenger RNA. Since our cDNA sequence matches with that of the genomic sequence we conclude that our TNF-β mRNA must be transcribed from the genomic sequence described here. Second, Abraham et al. [21] and Messer et al. [20] have reported that there are two types of TNF-β gene detectable by *Nco*I restriction frag-

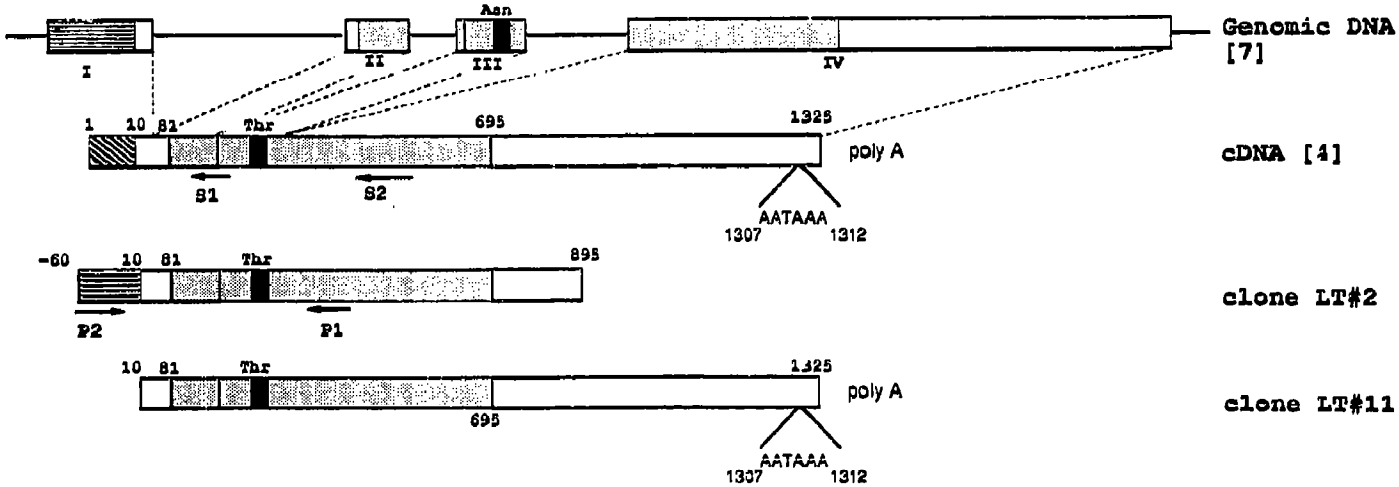
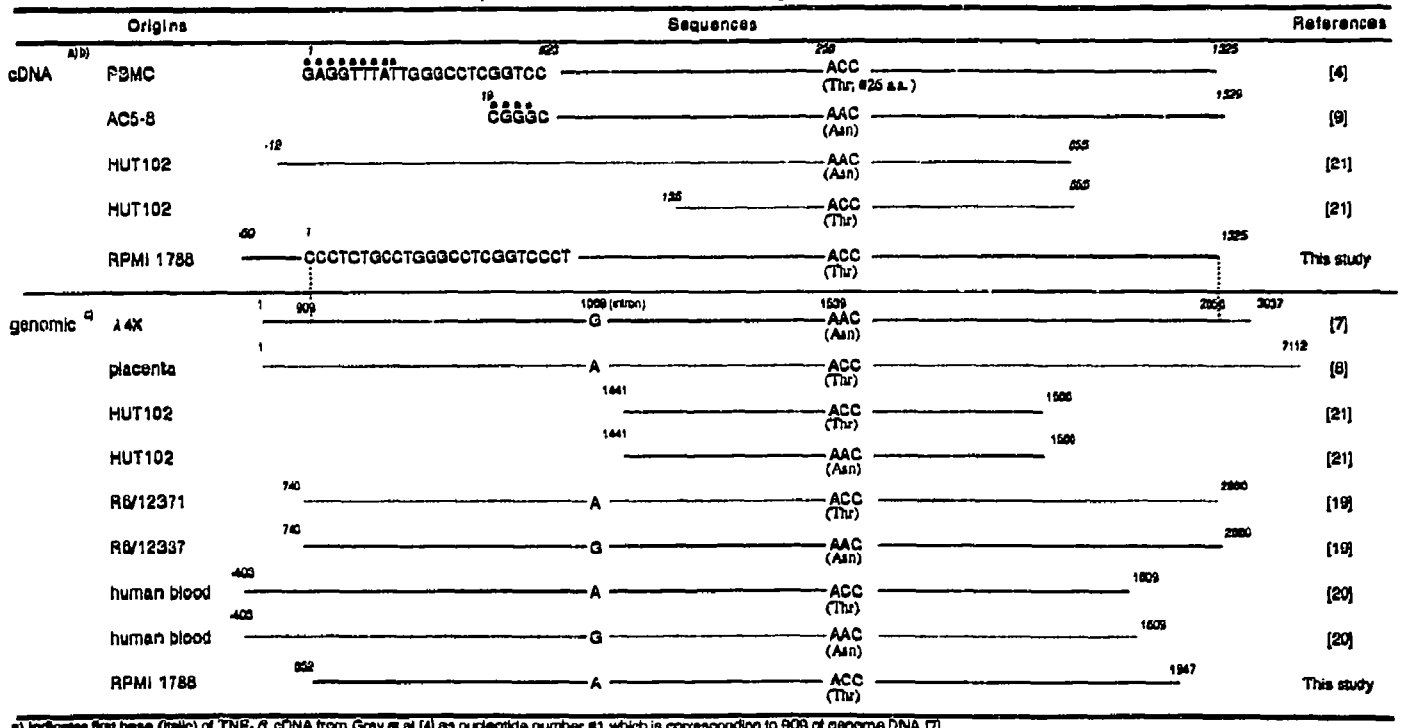


Fig. 1. Comparison of our TNF-β cDNA clones with published sequences. The first numbered position is nucleotide G of an initial base of a clone of Gray's [4] which corresponds to 909 of the genomic DNA [7]. The arrows indicate probes and primers. ■ shows AAC (Asn) or ACC (Thr) at amino acid residue 26; ▤ and ▧ indicate the differences among DNA sequences; ▩ indicates an open reading frame of TNF-β.

Table 1. Comparison of TNF-β cDNA and genomic DNAs.



a) Indicates first base (italic) of TNF-β cDNA from Gray et al [4] as nucleotide number #1 which is corresponding to 909 of genome DNA [7].
b) #23 C is deleted in [4].
c) Indicates first base of genomes TNF-β gene from Nedwin et al [7] as nucleotide number#1.
d) Dotted sequence indicated the base which is not matched in the genomic sequence [7].

ment length polymorphism (RFLP): the Asn (aa 26) type of TNF-β has an *Nco*I site (G at intron 1069), while the Thr type is not digested with *Nco*I because of an A instead of a G at the same position. Our PCR-amplified DNA could not be digested with *Nco*I at all (data not shown) and therefore the TNF-β gene of the RPMI 1788 cell line must be homozygous. Thirdly, the sequence of the cDNA and that of the genomic DNA originating from the same source (HUT102 cell line) were reported by Kato et al. [21], and, according to their data, the TNF-β gene in HUT102 appears to be heterologous; however, they did not determine the sequence of the region covering position 1069 which decisively indicates whether or not the gene is heterologous or homologous, depending on whether the site is an A or G. On the other hand our results proved that the TNF-β gene in the RPMI 1788 cell line is the homologous Thr type by cDNA and genomic DNA sequence analysis.

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