

Cloning and expression in *Escherichia coli* of the cDNA for murine tumor necrosis factor

(cDNA sequence/protein homology/macrophage cytotoxin/necrosin)

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ABSTRACT A murine tumor necrosis factor (MuTNF) cDNA was isolated from a cDNA library prepared by using mRNA from the murine macrophage-like cell line PU5-1.8 induced with 4 β -phorbol 12 β -myristate 13 α -acetate. The cDNA encodes a polypeptide consisting of a 79 amino acid pre sequence followed by a mature MuTNF sequence of 156 amino acids. The 235 amino acid murine pre-TNF polypeptide is 79% homologous to the human pre-TNF protein. There is one potential N-linked glycosylation site on MuTNF, in contrast to human TNF, which lacks any such site. The MuTNF cDNA, when engineered for expression in *Escherichia coli*, was found to direct the synthesis of biologically active MuTNF as determined by its cytotoxicity against several transformed cell lines.

Murine tumor necrosis factor (MuTNF) was originally identified in the serum of mice infected with *Mycobacterium bovis* strain BCG (bacillus Calmette–Guerin) and later injected with endotoxin (1, 2). One characteristic activity of this protein is that it is cytotoxic for tumor cells *in vitro* without killing normal cells (1–5). In addition, this factor has also been effective in causing hemorrhagic necrosis of the transplanted Meth A sarcoma in syngeneic mice (1, 2). It has been established that activated macrophages are the cellular source of tumor necrosis factor (TNF) (1, 4).

No species specificity has been observed for the TNF activities isolated from such sources as rabbits, mice, rats, and humans (6–9). Therefore, a comparison of the amino acid sequences of murine and human TNF (HuTNF) might elucidate the features shared by these two proteins that are essential for cytotoxic activity. In addition, murine model systems have been useful in the study of potential antitumor agents, and the availability of purified MuTNF along with HuTNF might prove valuable in the evaluation of the clinical potential of HuTNF.

Estimated values of the size of MuTNF range from M_r 15,000 to M_r 150,000 (10–13). Partially purified preparations of TNF isolated from the serum of mice treated with BCG and endotoxin have been shown to be glycoprotein in nature and to contain sialic acid and galactosamine (13). Recently, Kull and Cuatrecasas (11) reported the purification of a murine cytotoxin they have called necrosin from cell-free supernatants of a lipopolysaccharide (LPS)-induced murine macrophage cell line. Two prominent active forms of M_r 70,000 and 55,000 were isolated and found to be composed of non-sulfhydryl-linked aggregates of a M_r 15,000 monomer. Because of the similarities in their sizes and biological properties, it is likely that necrosin is identical to the protein we are calling MuTNF.

We recently reported the isolation and expression in *Escherichia coli* of the cDNA for HuTNF (9). HuTNF is a

protein of 157 amino acids that is initially synthesized as part of a 223 amino acid precursor. We demonstrated that HuTNF has cytotoxic activity in the murine L-929 *in vitro* assay and has *in vivo* tumor necrosis activity in the Meth A sarcoma tumor model (9).

In an effort to determine the structure of MuTNF and elucidate some of its characteristics, we isolated a MuTNF cDNA clone from a recombinant λ phage library prepared from the macrophage cell line PU5-1.8 and engineered it for expression in *E. coli*. We report here the structure of MuTNF and compare its sequence with that of HuTNF.

MATERIALS AND METHODS

Cell Lines and *in Vitro* TNF Assay. The murine macrophage cell lines used in these studies were obtained from the American Type Culture Collection (ATCC). P388D₁ (TIB63), was derived from the mouse strain DBA/2, and RAW 264.7 (TIB71) and WR19M.1 (TIB70) are cell lines derived from a BALB/c tumor induced by Abelson leukemia virus. WEHI-3 (TIB68) is a BALB/c-derived tumor cell line that is thought to represent an early stage in monocyte-myelocyte differentiation (14). J774A.1 is a BALB/c-derived macrophage cell line and PU5-1.8 (TIB61) is a monocytic tumor cell line (15), adapted to culture from a spontaneous lymphoid tumor of BALB/c mice. The media and culture conditions of all cell lines were as recommended by the ATCC. The human RPMI 1788 lymphoblastoid cell line used for RNA extractions was also obtained from the ATCC. Samples were assayed for cytolytic activity on mouse L-929 fibroblasts as described previously (16).

For the *in vitro* cytotoxicity assays we tested three human tumor cell lines, BT-20 (derived from a breast carcinoma), A549 (derived from a lung carcinoma), and ME-130 (derived from a cervical carcinoma), and one mouse tumor cell line, CMS-16 (derived from a mouse sarcoma). Briefly, 10⁴ units of either MuTNF or HuTNF was added to the wells of a 96-well microtiter plate and 2-fold serial dilutions were made. Approximately 5 \times 10⁴ cells were seeded into each well in a final volume of 200 μ l. After 72 hr the plates were stained with crystal violet and the absorbance was determined with a Microelisa plate reader (MR580, Dynatech). Percent viability was determined on the basis of a comparison between TNF-treated and untreated cells.

RNA Isolation and Blot Hybridization and cDNA Cloning. The PU5-1.8 cell line was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% L-glutamine and was adjusted to a final concentration of 1 \times 10⁶ cells per ml. The tumor-promoting agent 4 β -phorbol 12 β -myristate 13 α -acetate (PMA) was added at a concentration of

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Abbreviations: TNF, tumor necrosis factor; MuTNF, murine TNF; HuTNF, human TNF; HuLT, human lymphotoxin; bp, base pair(s); BCG, bacillus Calmette–Guerin strain of *Mycobacterium bovis*; PMA, 4 β -phorbol 12 β -myristate 13 α -acetate.

500 ng/ml and total RNA was extracted 5 hr after PMA addition (17). The polyadenylated mRNA was isolated by oligo(dT)-cellulose chromatography. RNA blot hybridization analysis (18) was performed using previously described procedures (19).

Double-stranded cDNA was prepared by oligo(dT) priming (9), using 5 μ g of mRNA. Synthetic *Eco*RI adapters (19) were ligated to 300 ng of cDNA and fractionated on a 6% polyacrylamide gel. Twenty-five nanograms of the electroeluted cDNA >600 base pairs (bp) was ligated into λ gt10 (20). Plaque screening (21) and phage DNA preparation (22) were performed as previously described.

Expression of MuTNF cDNA in *E. coli*. DNA from the recombinant phage λ 6 was digested with *Eco*RI and electrophoresed on a 6% polyacrylamide gel, and the 1105-bp *Eco*RI fragment was recovered by electroelution. This 1105-bp fragment was digested with *Taq* I and *Sac* I to isolate a 303-bp fragment coding for amino acids 9–109 of the mature protein. A 494-bp *Sac* I/*Stu* I fragment corresponding to amino acids 110–156 was also obtained from the λ 6 recombinant phage. Two synthetic complementary deoxyoligonucleotides, 5'-CTAGAATTATGTTACGTTCTTCTCAGAACT-3' and 5'-CGAGTCTGAGAAGAAGACGTAACATAATT-3', designed to code for amino acids 1–8 of MuTNF and also containing an ATG translational initiation codon, were synthesized (23).

The plasmid pTNFtrp (9) was digested with *Hind*III and filled in by using deoxynucleoside triphosphates and the Klenow fragment of DNA polymerase I to create a blunt end. The vector was then digested with *Xba* I and the large fragment was recovered by electroelution. The *Taq* I/*Sac* I fragment, *Sac* I/*Stu* I fragment, and the two synthetic deoxyoligonucleotides were inserted into the prepared pTNFtrp vector to give the final expression plasmid pMuTNFtrp by using previously described methods (17). *E. coli* 294/pMuTNFtrp was grown to 0.2 OD₅₅₀ unit in M9 medium containing carbenicillin at 20 μ g/ml. Induction of cells and preparation of cell extracts for assay of cytotoxic activity on L-929 cells have been previously described (9).

RESULTS

Identification of a MuTNF-Producing Cell Line. The human monocytic cell line HL-60 has been demonstrated to produce HuTNF after induction with the tumor-promoting agent PMA. This cell line provided a source of TNF protein and mRNA for amino acid sequencing (24) and cDNA cloning (9), respectively. Therefore we screened several murine monocyte-macrophage cell lines for their ability to produce a cytotoxic factor having similar properties to HuTNF. Cell-free supernatants from six cell lines were assayed for MuTNF production by using the L-929 *in vitro* assay system (Table 1). Supernatants from two of the cell lines, PU5-1.8 and RAW 264.7, were found to contain significant levels of a factor with TNF-like activity 4 hr after induction with various concentrations of PMA. This activity appears to be specifically induced by PMA, since low levels of cytotoxicity were observed from supernatants of uninduced cultures. Other investigators have also detected a cytotoxic activity from induced cultures of PU5-1.8 cells (25, 26). Since this cell line consistently produced the highest levels of the cytotoxic factor after PMA induction, it was selected for further testing.

To determine whether the cytotoxic activity observed in the PU5-1.8 cell line was the murine equivalent of HuTNF, human lymphotoxin (HuLT), or some unrelated cytotoxic molecule, antibody neutralization experiments were performed. When polyclonal antiserum prepared against either purified human TNF (unpublished results) or purified HuLT (16) was used, approximately 50% of the cytotoxic activity was neutralized. No further reduction in activity was ob-

Table 1. MuTNF production by murine macrophage cell lines induced by PMA

Cell line	MuTNF, units/ml			
	500 ng/ml	50 ng/ml	5 ng/ml	0 ng/ml
P388D ₁	<6	<6	<6	<6
PU5-1.8	1468	1504	260	22
J774A.1	<7	8	<7	<7
RAW 264.7	236	213	89	90
WR19M.1	57	32	20	20
WEHI-3	<6	<6	<6	<6

For each cell line four wells of a 24-well Linbro plate (no. 3524, Costar, Cambridge, MA) were seeded with 2 ml of the appropriate medium, containing 1×10^6 cells per ml. Immediately after seeding, PMA was added at a final concentration of 500, 50, 5, or 0 ng/ml to each of the four wells. Samples (250 μ l) were taken 4 hr after PMA addition and assayed for cytotoxic activity in the murine L-929 fibroblast assay (16).

served when HuTNF and HuLT antibodies were combined (data not shown).

Since the antibody neutralization results were ambiguous as to the relatedness of the murine cytotoxic activity to HuTNF or HuLT, RNA blot hybridizations were performed. A ³²P-labeled restriction fragment from the coding region of either the HuTNF or HuLT cDNA was used to probe filters containing PU5-1.8 mRNA. The human cell line RPMI 1788, which contains mRNA for both HuLT (27) and HuTNF (unpublished data), was used as a positive control for this experiment. As shown in Fig. 1, one strongly hybridizing band migrating at the position of 18S RNA is detected when RPMI 1788 mRNA is hybridized with a HuTNF probe and a 17S RNA species is detected with the HuLT probe.

mRNA isolated from the PU5-1.8 cell line was found to hybridize with the HuTNF probe but not with the HuLT probe. The major MuTNF-hybridizing band migrates at the position of 18S RNA. A weaker hybridizing signal is also observed migrating at the 28S position. When the same HuTNF probe was hybridized to a lane containing ribosomal RNA from the PU5-1.8 cell line, no detectable bands were observed at the 18S and 28S positions (data not shown), demonstrating that the signals observed with PU5-1.8 mRNA are in fact due to an mRNA species highly related to HuTNF mRNA.

MuTNF cDNA Clone Identification. To isolate a cDNA

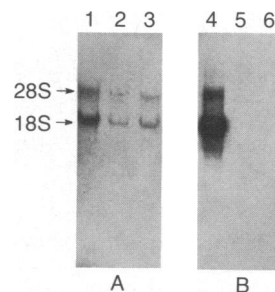


FIG. 1. Blot hybridization of mRNA derived from the murine PU5-1.8 and human RPMI 1788 cell lines. Lanes 1 and 4 contain 2 μ g of RPMI 1788 poly(A)⁺ mRNA isolated 4 hr after PMA induction; lanes 2 and 5 contain 2 μ g of 5-hr PMA-induced PU5-1.8 poly(A)⁺ mRNA; and lanes 3 and 6 contain 4 μ g of 5-hr PMA-induced PU5-1.8 poly(A)⁺ mRNA. The filter in A was hybridized with a 578-bp ³²P-labeled *Ava* I/*Hind*III restriction fragment from the human TNF cDNA plasmid (9). The filter in B was hybridized with a 650-bp *Eco*RI/*Pvu* II restriction fragment from the coding region of the human lymphotoxin cDNA (27). The migration positions of 28S and 18S ribosomal RNA are indicated to the left.

clone containing the sequence coding for MuTNF, a cDNA library was prepared using PU5-1.8 mRNA isolated 5 hr after PMA induction. An oligo(dT)-primed library of $\approx 400,000$ clones was constructed with the λ gt10 vector system (20) and screened with a HuTNF cDNA probe. A total of 95 hybridizing plaques was obtained, suggesting that approximately 0.025% of total PMA-induced PU5-1.8 mRNA codes for MuTNF. Ten of the plaques were purified and DNA was prepared from each. The phage $\lambda 6$ appeared to be the clone having the largest cDNA insert, as it contained two *EcoRI* restriction fragments 1105 and 533 bp in length. The sequence of the 1638-bp insert of $\lambda 6$ is presented in Fig. 2. The correct reading frame of MuTNF was determined by homology comparisons with the HuTNF sequence (9).

The 1638-bp cDNA contains a single open reading frame beginning with the first ATG codon encountered at the 5' end of the sequence (nucleotides 144–146). The sequence surrounding this methionine codon conforms to the consensus sequence shared by many mRNAs of higher eukaryotes (30). This ATG is preceded by termination codons in all three frames of the 140 nucleotides upstream, further suggesting that it serves as the site of translation initiation. The initiator codon is followed by 235 codons before a TGA termination triplet at nucleotides 849–851 is encountered. The 3' untranslated region of 786 nucleotides contains the hexanucleotide AATAAA (positions 1609–1614), which pre-

cedes the site of polyadenylation in many eukaryotic mRNAs (31).

On the basis of the homology with the NH₂-terminal sequence of HuTNF, we have designated the indicated leucine (Fig. 2) as amino acid number one of mature MuTNF. There are 79 amino acids preceding this leucine residue. The 76 amino acid pre sequence region of HuTNF is not observed on the mature HuTNF polypeptide and is probably involved in its secretion. On the basis of the fact that the pre sequence regions of MuTNF and HuTNF are highly conserved, one can infer that the 79 residues of MuTNF may also be involved in its secretion.

The mature MuTNF sequence of 156 amino acids contains cysteine residues at positions 69 and 100 and therefore has the potential to be stabilized by a disulfide bridge. From the DNA sequence we calculate the size of the mature MuTNF monomer to be M_r 17,260. Unlike HuTNF, there is one potential *N*-glycosylation site (32), located at asparagine-7 in MuTNF. The glycoprotein nature of a partially purified TNF isolated from the serum of BCG- and endotoxin-treated mice has been described and was reported to contain sialic acid and galactosamine (13).

Expression of MuTNF in *E. coli*. The procedure followed to directly express the cDNA insert of MuTNF in *E. coli* as a mature protein is outlined in *Materials and Methods*. The resulting MuTNF expression plasmid pMuTNF trp has the putative mature TNF coding sequence transcribed under the

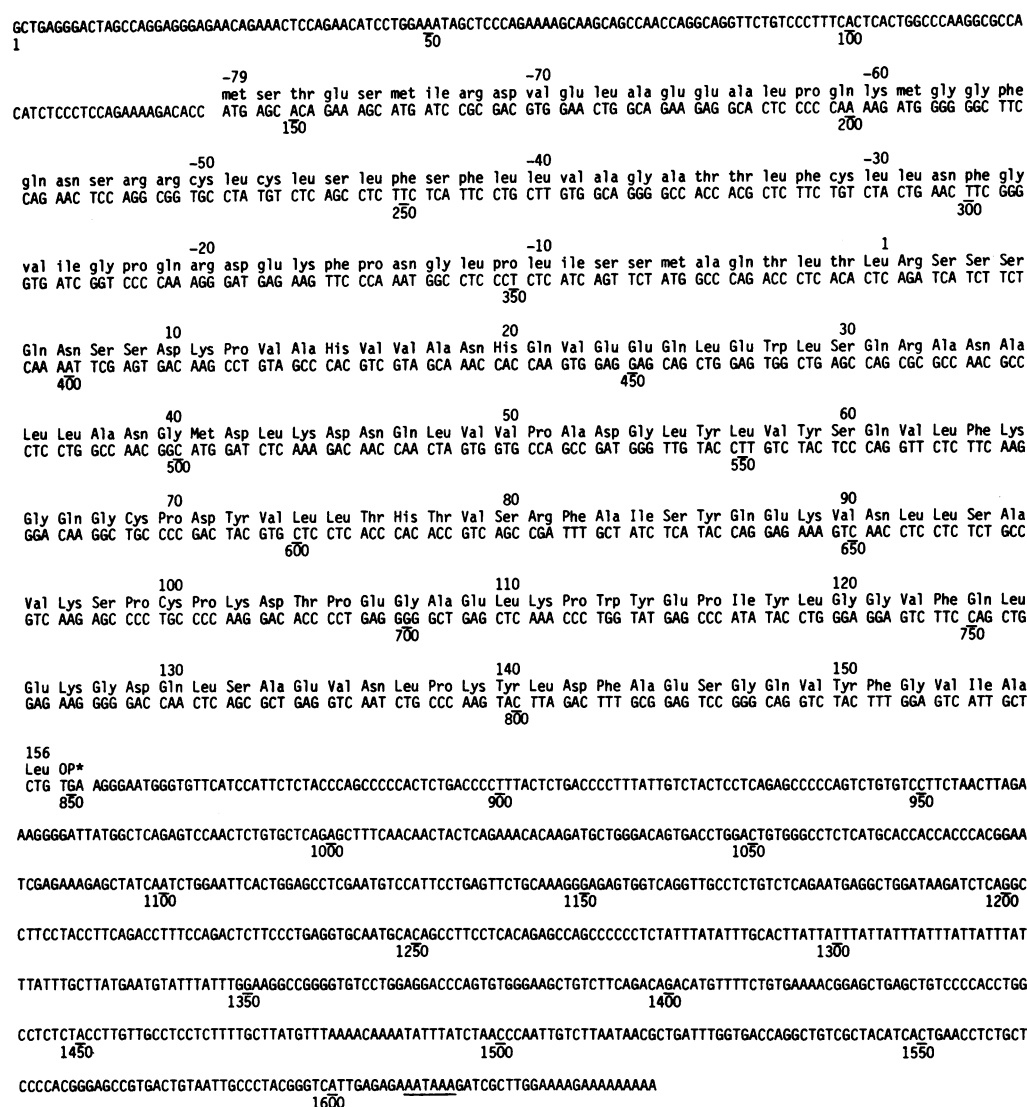


FIG. 2. Nucleotide sequence and deduced amino acid sequence of the MuTNF cDNA insert. The putative pre sequence is represented by the residues labeled -79 to -1 and are indicated by lowercase lettering. The entire sequence was determined by the dideoxynucleotide chain termination method (28) after subcloning fragments in the M13 vector mp7 (29). Numbers above each line refer to amino acid position and numbers beneath each line to nucleotide position. The underlined sequence indicates the polyadenylation recognition site. OP* indicates the opal chain termination signal.

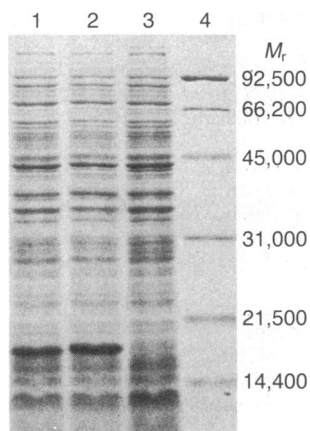


FIG. 3. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of MuTNF synthesized in *E. coli*. *E. coli* strain 294 transformed with pTNFtrp, pMuTNFtrp, or pBR322 was grown in M9 medium containing carbenicillin at 20 μ g/ml. Cultures were induced and samples were analyzed by sodium dodecyl sulfate/polyacrylamide slab gel electrophoresis as described previously (9). Lane 4 contains protein M_r standards, listed from the top: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. Lane 1, cell lysate of *E. coli* 294/pTNFtrp; lane 2, cell lysate of *E. coli* 294/pMuTNFtrp; lane 3, cell lysate of *E. coli* 294/pBR322.

control of the *E. coli trp* promoter. Extracts of *E. coli* 294/pMuTNFtrp were prepared and assayed for cytolytic activity. Approximately 1.2×10^6 units of activity was detected per ml of culture at $OD_{550} = 1$, whereas no activity was observed in control *E. coli* 294 cultures containing the pBR322 plasmid. Cultures of *E. coli* 294 transformed with pTNFtrp, the expression vector for HuTNF (9), gave 3.2×10^5 units per ml of culture.

A total extract of *E. coli* 294/pMuTNFtrp was analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. This sample contained a prominent polypeptide with an apparent M_r of 17,000 (Fig. 3, lane 2). This polypeptide also migrates with HuTNF synthesized in *E. coli* 294/pTNFtrp (lane 1). Densitometer scans of the gel indicate that MuTNF represents approximately 24% of the total protein in 294/pMuTNFtrp cultures, while HuTNF is about 15% of the total protein in cultures of 294/pTNFtrp. Therefore, it

appears that MuTNF has a specific activity about 2.3 times greater than HuTNF as measured in the murine L-929 assay.

To further investigate the possibility that TNF may display some species specificity, we assayed MuTNF and HuTNF for *in vitro* cytotoxicity on three human tumor cell lines and one additional murine tumor cell line. The results obtained indicate that HuTNF is 20–30 times more cytotoxic than MuTNF to the human cell lines BT-20, A549, and ME-180 (data not shown). In contrast, when assayed on the murine cell line CMS-16, MuTNF was found to be approximately 2 times as active as HuTNF. These results further suggest that some species specificity does exist between MuTNF and HuTNF, each being more cytotoxic on cell lines from the homologous species.

DISCUSSION

The PU5-1.8 murine macrophage cell line was found to synthesize MuTNF after induction with the tumor-promoting agent PMA. A full-length MuTNF cDNA clone was isolated from a PU5-1.8 cDNA library by hybridization with a HuTNF cDNA probe. The mature MuTNF encoded by this cDNA was synthesized in *E. coli* and exhibited the expected cytotoxic activity when assayed on murine L-929 cells.

A comparison of the deduced amino acid sequences of HuTNF and MuTNF is presented in Fig. 4. The overall homology of the precursor polypeptides is 79%, indicating that the murine and human TNF molecules are highly conserved. This high degree of homology with HuTNF is strong evidence that the polypeptide encoded by the cloned cDNA is indeed the murine equivalent of HuTNF. Definitive proof, however, will require *in vivo* testing using the Meth A sarcoma tumor necrosis assay.

The 79 amino acid pre sequence of MuTNF contains three more amino acids than the human sequence. Another difference is an additional amino acid in the human sequence at position 73. Both molecules contain two cysteine residues in their coding region at the same positions. There is evidence that cysteine-69 and cysteine-101 in HuTNF exist in a disulfide bond (24), suggesting that this may also be the case with MuTNF. A main structural difference between the two molecules is the lack of any potential *N*-glycosylation site on the human molecule. The MuTNF sequence contains one potential *N*-glycosylation sequence at amino acids 7–9 (Asn-Ser-Ser). This is consistent with the glycoprotein nature of

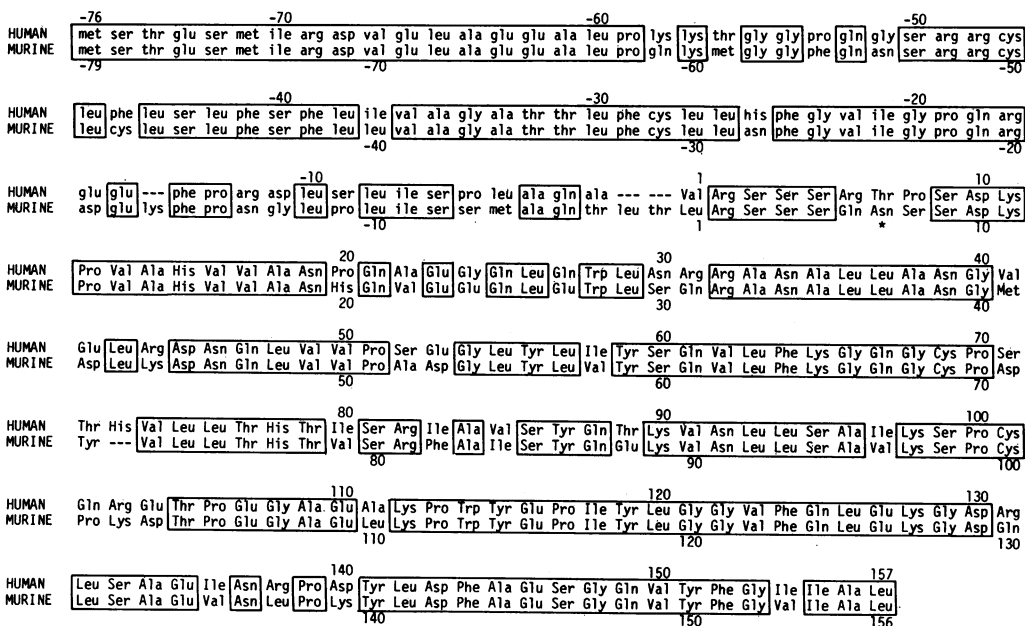


FIG. 4. Comparison of HuTNF and MuTNF sequences. Identical amino acids are boxed. The numbers above each row and below each row refer to amino acid positions of HuTNF and MuTNF, respectively. The pre sequence regions are numbered -76 to -1 and -79 to -1 for HuTNF and MuTNF, respectively, and are shown in lowercase lettering. The potential *N*-glycosylation site in MuTNF is indicated by an asterisk. The broken lines indicate amino acid deletions in the sequences.

partially purified MuTNF from the serum of mice treated with BCG and endotoxin (13). Although recombinant *E. coli*-derived nonglycosylated MuTNF exhibits potent cytotoxic activity *in vitro*, expression of MuTNF in mammalian cells should give an indication as to whether glycosylation does occur and has any additional significant effect on the biological activity of the molecule.

The first 18 amino acids of the pre sequence region are identical in HuTNF and MuTNF. This sequence is followed by two lysine residues in HuTNF and a single lysine in MuTNF, which could serve as cleavage sites for a trypsin-like protease. The conservation of these 18 amino acids could indicate that this peptide itself may have an important physiological function. Furthermore the long hydrophobic region in MuTNF at amino acids -19 to -47 and a similar homologous sequence in HuTNF could serve as a transmembrane domain. The 79 amino acid pre sequence of MuTNF is unusually long compared to most signal sequences, which are generally in the range of 20-30 amino acid residues (33). This atypical length may add support to the hypothesis that one or more stretches in this presequence region have a function or functions other than that involved in secretion of the molecule.

Recently, Kull and Cuatrecasas (11) reported the purification of a cytotoxin derived from lipopolysaccharide-induced J774.1, a murine macrophage-like cell line. They have named this factor necrosin and have shown that it exists in an aggregate form made up of M_r 15,000 monomers. They report that it has properties similar to those of the toxin isolated from other murine sources, such as tumor necrosis serum and the macrophage cell line RAW 264.1. It is possible that necrosin is identical to the product of the MuTNF cDNA we have cloned and expressed, but the definitive description of the relationship between these two proteins will have to await an analysis of the amino acid sequence of purified necrosin.

MuTNF appears to have a higher specific activity than HuTNF on murine cell lines, while the converse is true for human cell lines, suggesting that TNF does display some species specificity. As quantities of purified MuTNF become available it will be possible to test its activity in the *in vivo* Meth A sarcoma tumor necrosis assay (1). A comparison between the properties and activities of human and murine TNF *in vitro* and *in vivo* should then prove interesting.

Note Added in Proof. The *E. coli*-derived MuTNF has been purified to homogeneity and shown to be active in the *in vivo* Meth A sarcoma tumor necrosis assay.

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