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# Molecular cloning of mouse tumour necrosis factor cDNA and its eukaryotic expression

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ABSTRACT

Tumour necrosis factor (TNF), released by induced macrophages, causes tumour necrosis in animals and kills preferentially transformed cells in vitro. mRNA induced in the established mouse monocytic PU 5.1.8 cell line by lipopolysaccharide, was converted into double-stranded cDNA and cloned in the pAT153 vector. Recombinant plasmids were screened by plus-minus hybridization and TNF-specific oligonucleotide probes constructed on the basis of partial amino acid sequences of rabbit TNF. A series of TNF specific clones were identified and confirmed by hybrid selection of mouse TNF-specific mRNA. The sequence codes for a 235 amino acids long polypeptide, of which 156 amino acids presumably correspond to the mature product. It can be concluded that mature mouse TNF is a glycosylated dimer. Biologically active TNF was secreted by both Cos-I and CHO-cells transfected with the chimaeric expression vector pSV2d2-mTNF containing the coding region of the mouse TNF cDNA gene.

## INTRODUCTION

The antitumour effect of endotoxins has been known for over a century because of remarkable observations regarding spontaneous regression of certain human tumours following some bacterial infections (1,2). That tumour regression could be attributed to a serum factor elicited by endotoxin was proposed by Carswell et al. (3). TNF has so far mainly been prepared by in vivo induction of the protein in mice or rabbits. The best way to produce TNF was to inject the animals with Bacillus Calmette- Guérin (BCG) or Corynebacterium parvum, followed by endotoxin (lipopolysaccharide, LPS) 1 to 2 weeks later (3, 4, 5). Männel et al. (6), first reported in vitro induction of mouse TNF in macrophage-enriched populations derived from BCG infected mice, in peritoneal macrophages propagated with macrophage growth factor and in the macrophage-like tumour cell line PU 5.1.8. Partially purified TNF causes necrosis of some tumours in vivo and rather specifically kills many transformed cells in tissue culture. Upon purification, multiple size forms sharing similar physico-chemical properties such as heat stability and sensitivity towards proteases, but distinct in cytotoxic and necrotizing

activities can be segregated (7). Conclusive answers as to whether the in vitro cell killing effect of TNF is due to the same factor as that which causes haemorrhagic necrosis in vivo must await the unlimited availability of the pure product. Moreover, although the cell source of lymphotoxin (lymphocytes) and TNF (macrophages) is different, the mechanism of cytotoxic action, species near-independence, influence of growth inhibitors on the sensitivity of the cells, and stimulation of RNA synthesis in target cells (8) are shared by both products. Furthermore, the fairly high selectivity of both products for a variety of tumour cells (9) is most intriguing. The differences and similarities in gene structure, organization and biological properties of the two products will be better understood when the genes have been cloned and expressed. We describe here the characterization of mouse TNF mRNA and the cloning, molecular structure and eukaryotic expression of a plasmid coding for mouse TNF. The amino acid sequence of the mouse TNF protein, deduced from the nucleotide sequence analysis is presented. Very recently, the cloning and expression of human lymphotoxin cDNA (10) and of human TNF cDNA (11, 12) have been reported.

## MATERIALS AND METHODS

### In vitro induction of mouse TNF and TNF assay

Mouse TNF was induced in the mouse monocytic cell line PU 5.1.8 essentially as described by Männel et al. (6). The cells were grown in RPMI 1640 medium enriched with 10% FCS (low endotoxin content - 309 Gibco 011-6309) in roller bottles, stimulated at a concentration of $3.5 \times 10^6$ cells/ml with 5 µg LPS/ml in RPMI 1640 for 4 h and collected by centrifugation. TNF activity in the medium was assayed on L-929 cells in the presence of actinomycin D (13). Serial dilutions of the TNF-containing fraction were prepared in microtitre plates in Dulbecco medium - 10% FCS. Fifty thousand L-929 cells and actinomycin D to a final concentration of 1 µg/ml were added to each well. Incubation took place at 37°C or at 39.5°C for 18 h (the sensitivity is 2.5 times higher at 39.5°C than at 37°C). At the end of the incubation period, the cells were fixed, stained (0.5% crystal violet, 8% (v/v) formaldehyde (40%), 0.17% NaCl, 22.3% (v/v) ethanol) and counted. For certain experiments, stained cells were eluted in the microtitre plate in 33% acetic acid and the released dye was measured with a Kontron Spectrophotometer (577 nm). One TNF unit (U)/ml represents the reciprocal of the dilution of TNF required to reduce cell survivial by 50% within 18 h in the killing assay performed in the presence of actinomycin D.

Isolation of TNF mRNA and construction of a mouse cDNA library

For mRNA preparation, cells were lysed with 1% Nonidet-P40 in 0.1 M NaCl, 0.01 M Tris-HCl pH 8.5, 0.003 M $MgCl_2$, treated with proteinase K, extracted with phenol-chloroform-isoamyl alcohol (25:24:1), precipitated twice and purified over oligo-dT cellulose (Type 3-Collaborative Research). Poly $A^+$-RNA was further fractionated on a 5-20% sucrose gradient (Beckman SW41 rotor, 4°C, 40K, 19h) and TNF mRNA was identified by injecting aliquots of each fraction into Xenopus laevis oocytes (50 nl mRNA per oocyte ; 15 oocytes per sample) essentially as described by Devos et al. (14) except that the oocyte bathing medium also contained 1mM $CuSO_4$ and incubation time was reduced to 24 h. cDNA was prepared starting from 8 µg 17 S mRNA. Conditions for first-strand synthesis and subsequent removal of the RNA template was essentially as described by Devos et al. (15) except that for synthesis 750 U/ml human placental RNase inhibitor and 1000 U/ml AMV reverse transcriptase were used. Second-strand synthesis and $S_1$-nuclease treatment were carried out essentially as described by Maniatis et al. (16). Double stranded cDNA was size-fractionated on a Sepharose CL 4B column to enrich for fragments larger than approximately 500 bp. The resulting cDNA was inserted into the PstI site of pAT153 by G-C tailing and transformed into E.coli $DH_1$ ($\lambda$) according to Hanahan (17). The transformation mixture was plated on Millipore HATF (0.45 µM) filters that were layered on top of Luria broth agar plates containing 10 µg/ml tetracycline. Filters were stored on fresh plates also containing 20% glycerol at -20°C.

Screening of the mouse cDNA bank

Replicas of the mouse cDNA library were made according to Hanahan and Meselson (18). Two sets of replicas were screened by plus-minus hybridization; the plus probe being a $^{32}$P-labelled cDNA synthesized from the sucrose gradient fractions of mouse poly $A^+$-RNA which showed the highest TNF biological activity after oocyte injection, the minus probe being cDNA synthesized on an equivalent fraction obtained from uninduced cultures. Colony hybridization was essentially as described by Maniatis et al. (16). Plasmid DNA was extracted by the method of Birnboim and Doly (19), insert DNA was separated from the plasmid by restriction with PstI, followed by gel electrophoresis, and subsequently transferred and fixed on Millipore nitrocellulose filters as described by Southern (20). Partially overlapping probes were chemically synthesized (21, 22) and radioactively labelled to high specific activity ($>5 \times 10^8$ cpm/µg DNA) by a filling-in synthesis using Klenow polymerase in the presence of all four ($\alpha$)-$^{32}$P-labelled deoxynucleo-

side triphosphates. Heterologous hybridization was performed in 25 mM sodium phosphate buffer (pH 6.5) in the presence of 20% formamide at 42°C.

Hybridization-translation assay

Plasmid DNA of plus-minus-selected cDNA clones was isolated according to Pulleyblank et al. (23). Of each preparations about 30 ug was cleaved with the EcoRI restriction enzyme and bound to a membrane essentially as described by Kafatos et al. (24), except that Gene Screen (NEN, Boston, MA) was used instead of nitrocellulose filters, the ammonium acetate concentration increased from 1 M to 2 M, and the filters not baked but treated under UV light according to Church and Gilbert (25). The hybridization with TNF-positive poly $A^+$-RNA and subsequent RNA elution was essentially as described by Parnes et al. (26), except that the RNA was eluted in the presence of 5 ug poly $A^-$-RNA (oligo (dT)-cellulose run-through fraction) and that instead of a phenolization step, the RNA was precipitated twice with ethanol.

Insertion of the TNF cDNA sequence in an eukaryotic expression vector and transfection

The purified NarI-EcoRI fragment of the mouse cDNA gene (clone p-mTNF-3) was blunt-ended by the Klenow large fragment of E. coli polymerase I and provided with SalI linkers using T4-ligase. This fragment was then inserted in the unique SalI site of pSVd2-3. Cos-I cells were transfected with the recombinant expression vector essentially as described by Gheysen and Fiers (27), except that immediately after transfection, the cells were incubated for 4 h in medium containing 0.1 M chloroquine (28) and subsequently treated overnight with 1 mM sodium butyrate (29). Co-transfection of the CHO $DHFR^-$ cells with the recombinant expression vectors pSVd2-3 mTNF and pAdD26SV(A)-3 in a ratio of 10:1 was essentially as described by Scahill et al. (30).

## RESULTS

Construction of bacterial clones containing mouse TNF cDNA sequences

In vitro induced PU 5.1.8 cells were used for isolation of TNF-specific mRNA. The medium usually contained TNF activities of 4 - 6 x $10^3$ U/ml and about 10 mg of total RNA was obtained from $10^9$ lysed cells of which 2% represented polyadenylated (poly $A^+$)-RNA. This mRNA fraction was further enriched by sedimentation through a neutral 5 - 20% sucrose gradient (Fig. 1). The amount of TNF-specific mRNA was monitored by translation in Xenopus laevis oocytes followed by an assay of the TNF activity released in the bathing medium. The peak fraction migrated at 17 S and resulted upon

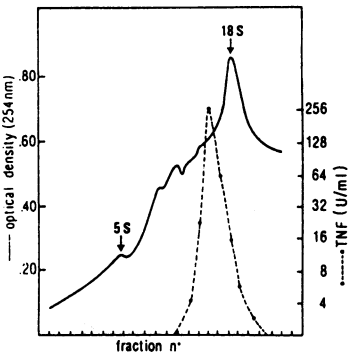



Fig. 1 Fractionation of TNF mRNA by sucrose density gradient sedimentation. LPS stimulated PU 5.1.8. cells were lysed with 1% Nonidet-P40, treated with proteinase K, extracted with phenol-chloroform-isoamylalcohol precipitated twice and purified over oligo-dT cellulose.Two hundred twenty-five µg of poly $A^+$-RNA was further fractionated on a 5-20% sucrose gradient. Fractions of 10 drops were collected, ethanol precipitated and the RNA dissolved in 20 ul $H_2O$. The RNA was translated in Xenopus laevis oocytes and the TNF activity in the bathing medium was measured on L-929 cells at 39.5°C in the presence of actinomycin D.

translation in 150-300 TNF U/ml. This fraction was used for the synthesis of double-stranded cDNA and cloning. Approximately 30,000 clones were obtained in this way of which 90% were ampicillin-sensitive indicating the presence of a cDNA insert.

Isolation and characterization of a mouse cDNA clone

About 5,000 colonies of the mouse cDNA library were subjected to a plus-minus hybridization screening to identify clones containing an induction-specific cDNA insert. About 2% of the clones hybridized with the plus probe only. These positives were chosen for a second round of plus-minus hybridization. In this way, 55 mouse clones corresponding to induced genes were finally selected. Plasmid DNA was individually extracted from these 55 plus-minus preselected cDNA clones and further pooled into 11 groups of 5 clones. The insert DNAs were separated from the plasmids by cleavage using the PstI restriction enzyme followed by agarose gel electrophoresis, and were subsequently transferred and fixed on nitrocellulose filter. Two sets of partially overlapping probes (Fig. 2, fragments 3 and 4) (31), corresponding to the known amino acid sequence of two CNBr-fragments derived from highly purified rabbit TNF (described elsewhere), were chemically synthesized and radioactively labelled by filling-in with Klenow polymerase in the presence of $(\alpha)$-$^{32}$P-nucleoside triphosphates. Duplicate filters were hybridized with probe 3 or 4, respectively. One group gave a positive hybridization signal with the probe derived from the rabbit TNF protein CNBr-fragment 3, and this result was confirmed at the individual clone level (in fact two individual cDNA clones were found positive in this group, but they turned out to be identical). This clone is further indicated as p-mTNF-1. Two additional mouse

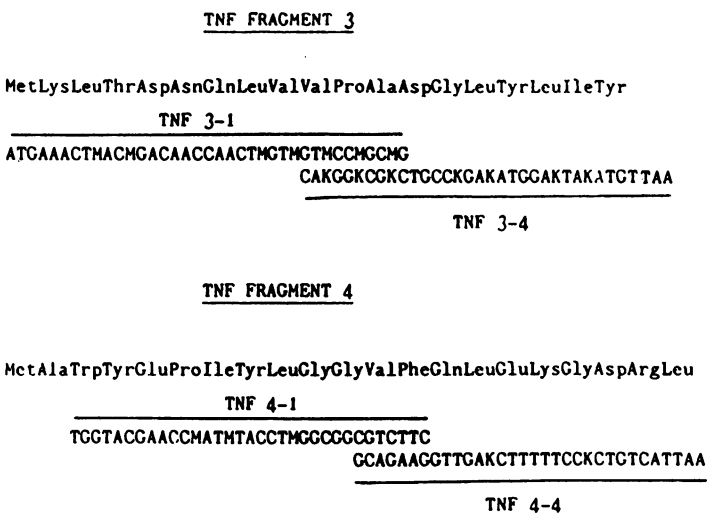



Fig. 2 Partial amino acid sequences of two CNBr-fragments (3 and 4) of highly purified rabbit TNF. Two partially overlapping nucleotide probes (TNF 3-1 and TNF 3-4; TNF 4-1 and TNF 4-4, respectively), coding for the indicated amino acid sequence, were synthesized and radioactively labelled to high specific activity by filling-in synthesis using Klenow polymerase in the presence of all four ($\alpha$)-$^{32}$P-labelled deoxynucleoside triphosphates.

TNF-specific cDNA clones (p-mTNF-2 and -3) were identified among the 55 plus-minus selected clones by hybridization with a nick-translated ($\alpha$)-$^{32}$P-labelled RsaI-fragment derived from the insert DNA of p-mTNF-1 (Fig. 4a).

Experiments showing that mouse cDNA clones specifically hybridized to TNF mRNA further demonstrated that the clones, selected by heterologous hybridization, in fact, contained TNF-specific sequences. Plasmid DNA was isolated from pools of the 55 plus-minus selected cDNA clones, each pool containing 6 or 7 individual clones. Approximately 30 ug of each preparation was cleaved with the EcoRI restriction enzyme, bound to a membrane and hybridized with TNF-positive poly A$^+$-RNA. Eluted RNA was assayed by injection in Xenopus laevis oocytes. Three pools scored positive in this hybridization-translation assay. These same pools turned out to contain p-mTNF-1, p-mTNF-2 and p-mTNF-3, respectively. Therefore, these three individual plasmids were tested again and all three were able to selectively hybridize with TNF-mRNA.

Restriction analysis indicated that all three cDNA clones (p-mTNF-1, -2

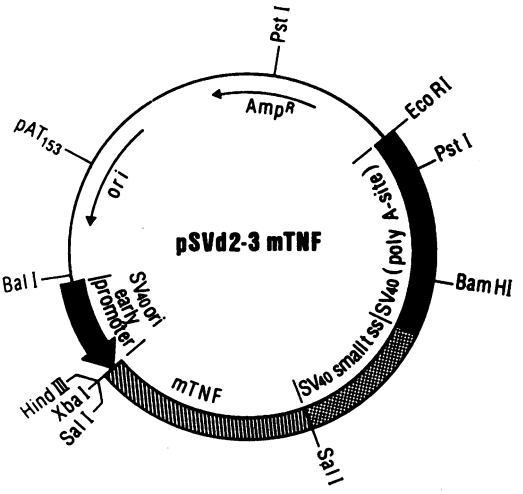



Fig. 3 pSV2d2-3, a modified pSV2 vector (35) was used for expression in eukaryotic cells; pSV2d2-3 contains the EcoRI-AvaI-fragment of pAT153 (poison-minus derivative), the PvuII-HindIII enhancer early promotor-ori-region of SV40 directly followed by the linker sequence HindIII-XbaI-SalI and followed by the SV40 small-t splicing signal and the SV40 polyadenylation region. The purified NarI-EcoRI fragment of the mouse cDNA gene (clone p-mTNF-3) was blunt-ended by the Klenow large fragment of E. coli polymerase I and provided with SalI linkers using T4-ligase. This fragment was then inserted in the unique SalI site of pSV2d2-3. Transfection of Cos-I and CHO cells was as described in Materials and Methods.

and -3) were derived from the same type of mRNA and, hence, almost certainly from the same gene. Digestion with PstI revealed that p-mTNF-1, 2 and -3 contained an insert of 1,550, 350 and 1,000 bp, respectively. The complete nucleotide sequence was deduced by sequencing both p-mTNF-1 and -3 using the Maxam-Gilbert procedure (32). Fig. 4a. shows a general outline of the cDNA gene and a map of some deduced and experimentally verified unique restriction sites.

Eukaryotic expression of mouse recombinant TNF

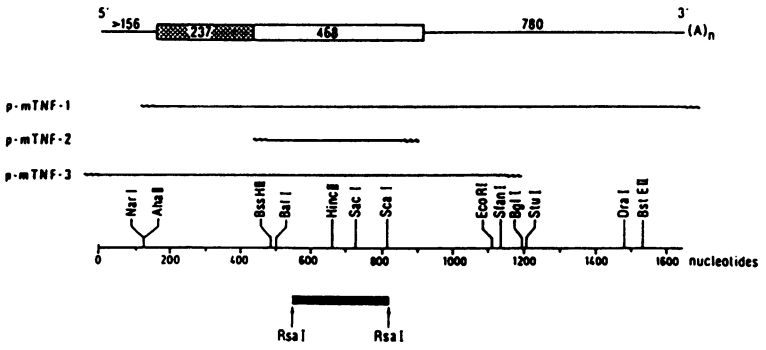
Final confirmation that the clone we had isolated does code for active TNF was shown by eukaryotic expression in both transfected Cos-I cells (33) and CHO cells (34) and by subsequent characterization of the resulting product in the L-929 assay system. For this purpose the NarI-EcoRI fragment of the mouse cDNA gene (clone p-mTNF-3) was purified and inserted in the unique SalI of the expression vector pSV2d2-3, a modified pSV2 vector (35). In the resulting chimaeric expression vector pSV2d2-3 mTNF (Fig. 3), the TNF cDNA sequence is under transcriptional control of the SV40 early promotor

sequence and followed by the SV40 small-t splicing signal and SV40 polyadenylation signal. Both orientations of the mouse TNF gene with respect to the SV40 early promotor were tested. In the samples taken 72 and 96 hours after transfection of the Cos-I cells, TNF activity was present in the supernatants of the sense-orientated construction but not in the supernatants of anti-sense constructions. Co-transformation of CHO DHFR$^-$ cells with pAdD26SV(A)-3 and pSV2d2-3 mTNF in a molar ratio of 1:10 and the subsequent selection of transformed cells by virtue of their DHFR$^+$ phenotype essentially as described by Scahill et al. (30) resulted in a set of transformants which secreted TNF activity (30 U/ml).

## DISCUSSION

Conditions have been optimized for induction of TNF in the mouse monocytic cell line PU 5.1.8. mRNA from the induced cells was then used as a source for molecular cloning. The resulting mouse cDNA library was first subjected to a plus-minus hybridization screening to identify clones containing an induction specific cDNA insert. Mouse TNF cDNA clones were identified among the preselected plus clones by hybridization with a TNF-oligonucleotide probe constructed on basis of partial amino acid sequences of rabbit TNF (purification and chemical characterization of rabbit TNF will be described elsewhere). Hybridization elution experiments confirmed the TNF specificity of the clones. Finally, proof that the isolated clone coded for TNF was obtained by biological expression. Transfection of Cos-I cells and CHO cells with pSVd2-3 mTNF containing the complete mouse cDNA TNF gene under control of the SV40 early promotor sequence led to secretion of TNF activity in the medium. Approximately 0.1% of the clones from the mouse cDNA bank hybridized to the internal mouse TNF-specific 252 basepair RsaI fragment (Fig. 4a). As the mRNA used for cloning was 10-15 fold enriched by sucrose gradient centrifugation, we estimate that approximately 1 out of 10,000-15,000 mRNA molecules is TNF-specific under the condition used for induction in PU 5.1.8. cells. The nucleotide sequence, shown in Fig. 4b, predicts a large open reading frame starting from the first ATG at position 157, specifying a polypeptide of 235 amino acids. The untranslated 5'-region in the cDNA is 156 nucleotides long but presumably incomplete considering the method used for the *in vitro* synthesis of double-stranded cDNA. The 3'-untranslated region is 780 nucleotides long and may be complete considering the presence of two potential AATAAA polyadenylation signals at

the end of the sequence. Based on amino-terminal amino acid sequence data obtained on highly purified human TNF secreted by phorbol ester-induced U-937 cells (manuscript in preparation), a presequence of 79 amino acids may be cleaved off between $Thr^{79}$-$Leu^{80}$ of the precursor to obtain the mature mouse TNF protein of 156 amino acids; the exact position of the N-terminal amino acid of mouse TNF, however, is not known and should be verified by direct characterization of the mature protein. A remarkably large homology exists in both the prepeptide (86%) and the mature protein (79%) region between the amino acid sequence of mouse and human TNF (described elsewhere). This large sequence homology between the two TNF proteins may be related to the nearly complete lack of species specificity of the biological action of TNF. The presequence (79 amino acids) is unusually long for a transmembrane signal; we do not yet know whether this is removed in a single step or whether parts of this segment fulfill a special role. Usually, a signal peptide is 20-30 amino acids long, contains one or more basic residues near the N-terminus and a highly hydrophobic part in the central region. The presequence contains a central region that is highly hydrophobic (from $Leu^{31}$ to $Ile^{56}$) but as many acidic as well as basic residues are present in the first 30 amino acids. Mouse TNF has been shown to be a glycoprotein (36), while human TNF is not (data not shown). Attachment of carbohydrate by N-glycosydic linkage is known to occur on the asparagine in the triplets Asp-X-Ser/Thr. Indeed, a potential N-glycosylation site of this type can be identified in mouse TNF at position 86 of the precursor sequence while this site is not conserved in the human sequence. The deduced mature mouse protein of 156 amino acids corresponds to a calculated $M_r$ of 17,200 (without the glycosyl group). Characterization of the mouse macrophage TNF product revealed that it has an apparent $M_r$ of 150,000 (7, 36) but this might be an aggregated form in the serum because partial purification in high ionic strength buffer allowed the identification of a $M_r$ 55,000 active protein on gel filtration (6). Gel filtration of human TNF under the same conditions revealed a protein of $M_r$ 35,000, while on a sodium dodecylsulphate-polyacrylamide gel, a $M_r$ 17,500 protein band could be identified (11). Therefore, it seems most likely that the native, mature, unglycosylated human TNF is a dimer, as well as the glycosylated mouse TNF. Analogous results with human interferon gamma were previously obtained, where it was shown that the mature protein also exists as a dimer (37). Two cysteines (at positions 148 and 179 in the precursor) may be involved in an intrasubunit disulphide bridge. Indeed the two subunits in the dimer

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   1  CCTCAGCGAGGACAGCAAGGGACTAGCCAGGAGGGAGAACAGAAACTCCAGAACATCCTG  60
                                                  H  S  A  E  S  H  I  R
  61  GAAATAGCTCCCAGAAAAGCAAGCAGCCAACCAGGCAGGTTCTGTCCCTTTCACTCACTG 120
 121  GCCCAAGGCGCCACATCTCCCTCCAGAAAAGACACCATGAGCACAGAAAGCATGATCCGC 180
                                                  D  V  E  L  A  E  E  A  L  P  Q  K  M  G  G  F  Q  N  S  R
 181  GACGTGGAACTGGCAGAAGAGGCACTCCCCCAAAAGATGGGGGGCTTCCAGAACTCCAGG 240
                                                  R  C  L  C  L  S  L  F  S  F  L  L  V  A  G  A  T  T  L  F
 241  CGGTGCCTATGTCTCAGCCTCTTCTCATTCCTGCTTGTGGCAGGGGCCACCACGCTCTTC 300
                                                  C  L  L  N  F  G  V  I  G  P  Q  R  D  E  K  F  P  N  G  L
 301  TGTCTACTGAACTTCGGGGTGATCGGTCCCCAAAGGGATGAGAAGTTCCCAAATGGCCTC 360
                                                  P  L  I  S  S  M  A  Q  T  L  T  L  R  S  S  S  Q  N  S  S
 361  CCTCTCATCAGTTCTATGGCCCAGACCTCACTCTCAGATCATCTTCTCAAAATTCGAGT  420
                                                  D  K  P  V  A  H  V  V  A  N  H  Q  V  E  E  Q  L  E  W  L
 421  GACAAGCCTGTAGCCCACGTCGTAGCAAACCACCAAGTGGAGGAGCAGCTGGAGTGGCTG 480
                                                  S  Q  R  A  N  A  L  L  A  N  G  M  D  L  K  D  N  Q  L  V
 481  AGCCAGCGCGCCAACGCCCTCCTGGCCAACGGCATGGATCTCAAAGACAACCAACTAGTG 540
                                                  V  P  A  D  G  L  Y  L  V  Y  S  Q  V  L  F  K  G  Q  G  C
 541  GTGCCAGCCGATGGGTTGTACCTTGTCTACTCCCAGGTTCTCTTCAAGGGACAAGGCTGC 600
                                                  P  D  T  V  L  L  T  H  T  I  S  R  F  A  I  S  Y  Q  E  K
 601  CCCGACTACGTGCTCCTCACCCACACCGTCAGCCGATTTGCTATCTCATACCAGGAGAAA 660
                                                  V  N  L  L  S  A  V  K  S  P  C  P  R  D  T  P  E  G  A  E
 661  GTCAACCTCCTCTCTGCCGTCAAGAGCCCCTGCCCCAGGGACACCCCTGAGGGGGCTGAG 720
                                                  L  K  P  W  Y  E  P  I  Y  L  G  G  V  F  Q  L  E  K  G  D
 721  CTCAAACCCTGGTATGAGCCCATATACCTGGGAGGAGTCTTCCAGCTGGAGAAGGGGGAC 780
                                                  Q  L  S  A  E  V  N  L  P  K  Y  L  D  F  A  E  S  G  Q  V
 781  CAACTCAGCGCTGAGGTCAATCTGCCCAAGTACTTAGACTTTGCGGAGTCCGGGCAGGTC 840
                                                  Y  F  G  V  I  A  L  TGA
 841  TACTTTGGAGTCATTGCTCTGTGAAGGGAATGGGTGTTCATCCATTCTCTACCCAGCCCC 900
 901  CACTCTGACCCCTTTACTCTGACCCCTTTATTGTCTACTCCTCAGAGCCCCCAGTCTGTG 960
 961  TCCTTCTAACTTAGAAAGGGGATTATGGCTCAGAGTCCAACTCTGTGCTCAGAGCTTTCA 1020
1021  ACAACTACTCAGAAACACAAGATGCTGGGACAGTGACCTGGACTGTGGGCCTCTCATGCA 1080
1081  CCACCATCAAGGACTCAAATGGGCTTTCCGAATTCACTGGAGCCTCGAATGTCCATTCCT 1140
1141  GAGTTCTGCAAAGGGAGAGTGGTCAGGTTGCCTCTGTCTCAGAATGAGGCTGGATAAGAT 1200
1201  CTCAGGCCTTCCTACCTTCAGACCTTTCCAGACTCTTCCCTGAGGTGCAATGCACAGCCT 1260
1261  TCCTCACAGAGCCAGCCCCCCTCTATTTATATTTGCACTTATTATTTATTATTTATTTAT 1320
1321  TATTTATTTATTTGCTTATGAATGTATTTATTTGGAAGGCCGGGGTGTCCTGGAGGACCC 1380
1381  AGTGTGGGAAGCTGTCTTCAGACAGACATGTTTTCTGTGAAAACGGAGCTGAGCTGTCCC 1440
1441  CACCTGGCCTCTCTACCTTGTTGCCTCCTCTTTTGCTTATGTTTAAAACAAAATATTTAT 1500
1501  CTAACCCAATTGTCTTAATAACGCTGATTTGGTGACCAGGCTGTCGCTACATCACTGAAC 1560
1561  CTCTGCTCCCCACGGGAGCCGTGACTGTAATTGCCCTACAGTCAATTGAGAGAAAAAAAG 1620
1621  ATCGCTTAAAAATAAAAAACCCCCC                                    1644
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presumably are not held together by covalent linkages as the same $M_r$ 17,500 band can be detected on a sodium dodecylsulphate-polyacrylamide gel under reducing as well as non-reducing conditions. Furthermore, mouse TNF activity is inactivated by treatment with dithiothreitol (7), suggesting that the cystine-bond might be required for biological activity. The isolation of a cDNA clone encoding mouse TNF opens many new possibilities for further study of this promising anti-cancer protein at a biochemical, molecular biological and biological level. Especially intriguing are its mechanism of action and the molecular basis for its apparent selective toxicity for transformed cells in vitro and tumour necrotizing activity in mice. It should also allow further investigation of the synergism with other lymphokines (38) and various drugs. Hopefully, these preclinical data may open the way to an evaluation of the potential application of human TNF in clinical medecine.
While this manuscript was being prepared, we learned of the results of Pennica et al. (12), and Shirai et al. (39), who recently reported the cloning and expression of human TNF. The nucleotide and amino-acid sequence which they published is in complete agreement with our results on human TNF (11).

Fig. 4a. Map of the mouse TNF mRNA and the isolated cDNA clones. The diagram at the top represents the structure of the mouse TNF mRNA. The stippled box indicates the corresponding putative signal peptide and the clear box indicates the coding region for the mature mouse TNF polypeptide. The estimated length in nucleotides of the 3'- and 5'-untranslated regions and the putative prepeptide and mature protein coding sequences are also given. $(A)_n$ indicates the 3'-poly(A)-tail. Lines underneath represent the regions of the mouse TNF mRNA covered by the three isolated mouse cDNA clones p-mTNF-1, -2 and -3, respectively. Wavy lines indicate the homopolymer tails used to construct the cDNA clones. At the bottom, a map of most unique restriction sites constructed from a computer search made on the complete nucleotide sequence is presented. Also indicated (black bar) is the RsaI fragment which was purified and used in further hybridization experiments.
Fig. 4b. Nucleotide sequence of the mouse TNF cDNA and its derived amino acid sequence of the pre-TNF polypeptide. A continuous nucleotide sequence of 1644 nucleotides is presented. Full boxes indicate the start and stop codon, dashed boxes indicate two putative AATAAA polyadenylation signals. Amino acids are given in one letter code. The presequence is underlined (the putative N-terminus has been chosen on the basis of analogy with the human mature TNF sequence, indicated by an arrowhead). A glycosylation signal corresponding to the sequence N-X-S/T, and two cysteine residues, believed to be involved in an intramolecular disulphide bridge, are also indicated in full boxes.

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Abbreviations : BCG, Bacillus Calmette-Guérin; bp, base pairs; FCS, foetal calf serum; LPS, lipopolysaccharide; $M_r$, molecular weight; poly $A^+$, polyadenylated; TNF, tumour necrosis factor; U, unit.

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