



The present invention relates to a TNF receptor and to a TNF binding protein.

Tumor necrosis factor (TNF- $\alpha$ ) was found for the first time in the serum of mice and rabbits which had been infected with Bacillus Calmette-Guerin and had endotoxins injected thereinto, and was recognized because of its cytotoxic and antitumor characteristics (Carswell et al., 1975). It is above all produced by activated macrophages and monocytes. Numerous cell types which are the targets for TNF have surface receptors with a high affinity for this polypeptide (Old et al., 1987); it was assumed that lymphotoxin (TNF- $\beta$ ) binds to the same receptor (Aggarwal et al., 1985, Gullberg et al., 1987). TNF- $\alpha$  is identical with a factor designated as cachectin (Beutler et al., 1985) which suppresses lipoprotein lipase and leads to hypertriglyceridemia in the case of chronic inflammatory and malign diseases (Torti et al., 1985, Mahoney et al., 1985). TNF- $\alpha$  takes probably part in the regulation of growth and in the differentiation and function of cells which play a role in inflammations, immune processes and hematopoiesis.

TNF can exert a positive effect on the host organism by stimulation of neutrophils (Shalaby et al., 1985, Klebanoff et al., 1986) and monocytes and by inhibition of the replication of viruses (Mestan et al., 1986, Wong et al., 1986). Moreover, TNF- $\alpha$  activates the immune defense against parasites and acts directly and/or indirectly as a mediator in immunoreactions, inflammatory

processes and other processes in the organism, the mode of action being not yet clear in many instances.)

The administration of TNF- $\alpha$  (Cerami et al., 1988) may, however, be accompanied by harmful phenomena (Tracey et al., 1986), such as shock and tissue damage which can be counteracted by antibodies against TNF- $\alpha$  (Tracey et al., 1987). A number of observations suggest that endogenously released TNF- $\alpha$  plays a role in various pathologic states. For instance, TNF- $\alpha$  seems to be a mediator of cachexia which arises in chronic-invasive, for instance parasitary diseases. TNF- $\alpha$  also seems to play an important role in the pathogenesis of shocks caused by Gram-negative bacteria (endotoxin shock); it might take part in some, though not all, effects of lipopolysaccharides (Beutler et al., 1988). Likewise, it has been claimed that TNF plays some part in tissue damage occurring within the frame of inflammatory processes in joints and other tissues and in the lethality and morbidity of the graft-versus-host reaction (GHVR, graft rejection (Piguet et al., 1987). Moreover, a connection between the concentration of TNF in the serum and the deadly end of meningococcal diseases has been reported on (Waage et al, 1987).

Further, it has been found that the administration of TNF- $\alpha$  over a long period of time causes a state of anorexia and tabes with similar symptoms as cachexia which is accompanied by neoplastic and chronic infectious diseases (Oliff et al., 1987).

There have been reports on a TNF-inhibiting activity of a protein from the urine of febrile patients whose effect is supposed to be due to a competitive mechanism at the receptor plane itself (Seckinger et al., 1988) (similar to the effect of the interleukin-1 inhibitor (Seckinger et al., 1987)).

EP-A2 308 378 describes a TNF inhibiting protein obtained from human urine. Its activity was detected in the urine of healthy and sick persons and determined because of the ability to inhibit the binding of TNF- $\alpha$  to its receptors on human HeLa cells and FS 11 fibroblasts and also the cytotoxic effect of TNF- $\alpha$  on murine A9 cells. This protein was substantially purified to homogeneity and characterized by its N-terminus. Although two basically possible methods are shown in this patent publication for obtaining the DNA coding for the protein and the recombinant protein, no specific information is given as to which one of the theoretically possible solutions is the successful one.

[A protein that inhibits the biological effects of TNF- $\alpha$  by preventing the binding of TNF- $\alpha$  to its cell surface receptor through interaction with TNF- $\alpha$  could be identified in the dialysis urine of uremia patients in preliminary tests regarding the present invention. An affinity to TNF- $\beta$  was also detected in this protein. 7

The presence of this protein (hereinafter called TNF-BP) in concentrated dialysis urine was detected by competition with the binding of radioactively labeled recombinant TNF- $\alpha$  to a subclone of HL-60 cells, with

measurements being made of the influence of dialyzed urine on the binding of  $^{125}\text{I}$ -TNF- $\alpha$  to the cells. The binding tests as performed revealed a dose-dependent inhibition of the TNF- $\alpha$  binding to the cell by concentrated dialysis urine (the possible interpretation that the observed decrease in binding might be caused by TNF- $\alpha$  itself which possibly exists in urine, or by TNF- $\beta$  which competes for the binding, was ruled out by the finding that the decrease in binding could not be eliminated by use of TNF- $\alpha$  and TNF- $\beta$  antibodies).

By analogy, proof could be furnished in preliminary tests regarding the present invention that TNF-BP has also an affinity to TNF- $\beta$ ; it amounts to about 1/50 of its affinity to TNF- $\alpha$ .

It was found by means of gel chromatography on Sephacryl 200 that a substance in the urine and serum of dialysis patients and in the serum of healthy persons with recombinant TNF- $\alpha$  forms a complex having a molecular weight of about 75,000.

TNF-BP was enriched from several samples of dialysis urine of uremia patients by partial purification by means of pressure ultrafiltration, ion exchange chromatography and gel chromatography 62 times.

The resultant preparations were used for detecting the biological activity of TNF-BP by inhibiting the growth-inhibiting effect of TNF- $\alpha$  on HL-60-10 cells. A dose-dependent impact of TNF-BP on the biological activity of TNF- $\alpha$  was found. Furthermore, an examination was

conducted as to the binding characteristics of cells by pretreatment with TNF-BP and excluding competition binding assay. Proof could be furnished that pretreatment of the cells with TNF-BP does not impair the binding of TNF- $\alpha$  to the cell. This shows that the effect of TNF-BP is not based on its possible binding to the cell and competition with TNF- $\alpha$  for the binding to the receptor.

The substantially homogeneous protein was obtained in highly purified form by concentrating the urine from dialysis patients by ultrafiltration, dialyzing the concentrated urine and enriching it in a first purification step by means of DEAE Sephacel chromatography to four times its amount. Further enrichment took place by means of affinity chromatography by TNF- $\alpha$  bound to Sepharose. Final purification was performed by means of reversed phase chromatography (FPLC).

It could be shown that the substantially highly purified protein inhibits the cytotoxic effect of TNF- $\alpha$  on WEHI 164 clone 13 cells (Olsson et al., 1989).

The N-terminal amino acid sequence could be determined with respect to the substantially highly purified protein. It was defined as Asp-Ser-Val-Xaa-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-(main sequence)(in addition the following N-terminal sequence was found in traces: Leu-(Val)-(Pro)-(His)-Leu-Gly-Xaa-Arg-Glu-(side sequence)). A comparison of the main sequence with the N-terminal sequence of the TNF inhibiting protein as disclosed in EP-A2 308 378 shows identity between the two proteins.

The following amino acid composition was determined, indicated in mol of amino acid per mol of protein and in mol % of amino acid determined as the mean value of a hydrolysis for 24 and 48 hours:

|           | Mol amino acid/<br>mol protein | mol %<br>amino acid |
|-----------|--------------------------------|---------------------|
| -----     |                                |                     |
| Asp + Asn | 27.5                           | 10.9                |
| Thr       | 15.8                           | 6.3                 |
| Ser       | 20.7                           | 8.2                 |
| Glu + Gln | 35.0                           | 13.8                |
| Pro       | 9.5                            | 3.8                 |
| Gly       | 16.0                           | 6.3                 |
| Ala       | 4.2                            | 1.7                 |
| Cys       | 32.3                           | 12.8                |
| Val       | 10.8                           | 4.3                 |
| Met       | 1.1                            | 0.4                 |
| Ile       | 7.0                            | 2.8                 |
| Leu       | 20.2                           | 8.0                 |
| Tyr       | 6.1                            | 2.4                 |
| Phe       | 8.1                            | 3.2                 |
| His       | 11.1                           | 4.4                 |
| Lys       | 15.7                           | 6.2                 |
| Arg       | 11.8                           | 4.7                 |
| -----     |                                |                     |
| Total     | 252.9                          | 100                 |
| =====     |                                |                     |

A content of glucosamine was detected by means of amino acid analysis. The results of an affinoblot carried out

with Concanavalin A and wheat germ lectin also showed that TNF-BP is a glycoprotein.

The substantially homogeneous protein was tryptically digested and the amino acid sequences were determined out of 17 of the cleavage peptides obtained. Furthermore, the C-terminus was analyzed.

TNF-BP has obviously the function of a regulator of the TNF activity with the ability to buffer the changes in concentration of free, biologically active TNF- $\alpha$ . TNF-BP might also influence the excretion of TNF by the kidney because the complex formed with TNF, whose molecular weight was determined by means of gel permeation chromatography on Sephadex G 75 with about 75,000 was obviously not retained by the glomerulus, in contrast to TNF.

The TNF-BP was identified from the urine of dialysis patients as one of three main protein components that show affinity to TNF and which jointly elute with TNF-BP from the TNF affinity chromatography column. The two other proteins, however, obviously bind in a manner which does not impair the binding of TNF- $\alpha$  to its cell surface receptor.

The results obtained with respect to the biological activity of the TNF-BP, in particular the comparison of the binding constant with the binding constant described for the TNF receptor (Creasey et al., 1987) are a first sign that this protein might be the soluble part of a TNF receptor.

Because of its ability to inhibit the biological activity of TNF- $\alpha$  and TNF- $\beta$ , the TNF-binding protein is suited for use at indications in which a reduction of the TNF activity in the organism is recommended. Derivatives or fragments of the TNF binding protein having the ability to inhibit the biological activity of TNF are also suited for use in the case of such indications.

TNF-BP (or its functional derivatives or active fragments) can be used for the prophylactic and therapeutic treatment of the human or animal body in the case of indications in which a detrimental effect of TNF- $\alpha$  takes place. These diseases include, in particular, inflammatory and infectious and parasitary diseases or shocks wherein endogeneous TNF- $\alpha$  is released, further cachexia, GVHR, ARDS (Adult Respiratory Distress Symptom) and autoimmune diseases such as rheumatoid arthritis, etc. These also include pathological states which may occur as side effects in the treatment with TNF- $\alpha$ , especially in case of high dosage, e.g. serious hypotension or disturbance of the central nerve system.

Because of its TNF-binding properties, TNF-BP is also suited as a diagnostic agent for the determination of TNF- $\alpha$  and/or TNF- $\beta$ , e.g. as one of the components in radioimmunoassays or enzyme immunoassays, optionally together with antibodies against TNF.

Because of its properties, this protein is a pharmacologically valuable active substance which being

from natural sources cannot be represented in sufficient amounts by means of protein-chemical methods.

There has therefore been a need to produce this protein (or related proteins with the ability to bind TNF) in a recombinant manner to provide it in sufficient amounts for therapeutic applications.

"Ability to bind TNF" is defined within the scope of the present invention as the property of a protein to bind to TNF- $\alpha$  such that the binding of TNF- $\alpha$  to the functional part of the receptor is prevented and the effect of TNF- $\alpha$  is inhibited or counteracted in human or animal organisms. This definition includes the ability of a protein to bind to other proteins, e.g. to TNF- $\beta$ , and to inhibit the activity thereof.

It has been the object of the present invention to provide the DNA coding for TNF-BP so as to permit, on the basis thereof, the preparation of recombinant DNA molecules with the aid of which suitable host organisms can be transformed for producing TNF-BP or functional derivatives or fragments thereof.

It has also been the object to determine whether TNF-BP is the soluble part of a TNF receptor. This assumption has been confirmed, thereby creating the basis for the clarification of the receptor sequence.

Another object within the scope of the present invention has been the provision of the cDNA coding for a TNF receptor for producing recombinant human TNF receptor.

The presence of a specific receptor having a high affinity for TNF- $\alpha$  on different types of cells was shown by several teams. The isolation and preliminary characterization of a TNF- $\alpha$  receptor have recently been reported on for the first time (Stauber et al., 1988). Since the binding of radioactively labeled TNF- $\alpha$  can be undone by a surplus of TNF- $\beta$  (Aggarwal et al., 1985), the suggestion has been made that TNF- $\alpha$  and TNF- $\beta$  should have a common receptor. However, since it could be demonstrated that specific types of cells that are responsive to TNF- $\alpha$  are entirely or partly insensitive to TNF- $\beta$  (Locksley et al., 1987), doubts about the existence of a common receptor were again expressed.

By contrast, recent results on the binding characteristics of TNF- $\alpha$  to receptors seem to corroborate the theory of a common receptor again (Stauber et al., 1989), and it is pointed out in the paper that differences exist between TNF- $\alpha$  and TNF- $\beta$  with respect to the interaction with the receptor and also with respect to the events occurring in the cell after an interaction between ligand and receptor.

Another TNF-binding protein was recently reported on and it has been assumed that this is the soluble form of another TNF receptor (Engelmann et al., 1990).

The availability of the DNA coding for a TNF receptor is a prerequisite for the preparation of recombinant receptor and considerably facilitates, e.g., the conduction of comparative studies of different cell types as to their TNF- $\alpha$  and/or TNF- $\beta$ -receptor(s) and the

reactions initiated by the binding of TNF to the receptor in the cell. This, in turn, helps to clarify the three-dimensional structure of the receptor, thereby creating the precondition for a rational design for the development of agonists and antagonists of the TNF activity.

In solving the above-mentioned problems, it was assumed that the screening of cDNA libraries with the aid of hybridizing probes that are derived from amino acid sequences of short peptides might encounter major difficulties because of the degeneration of the genetic code. In addition, this procedure is rendered more difficult if one does not know with respect to a protein, such as TNF-BP, in which tissues it is synthesized. In case of failure of such a method it might then not be possible to ascertain for sure whether this is due to the selection of an unsuitable cDNA library or to the inadequate specificity of the hybridizing probes.

Therefore, to obtain the DNA coding for the TNF-BP, it was first proceeded as follows according to the invention:

A library of the fibrosarcoma cell line HS913 T which had been induced with TNF- $\alpha$  and was present in  $\lambda$  gt11 was used as the cDNA library. To obtain  $\lambda$  DNA with TNF-BP sequences from this library, the great sensitivity of the polymerase chain reaction (PCR, (Saiki, 1988)) had been exploited. (With the aid of this method it is possible to obtain from a whole cDNA library an unknown DNA sequence which is flanked by oligonucleotides designed on the

basis of known partial amino acid sequences and used as primers. Such a longer DNA fragment can then be used as a hybridizing probe, for instance for isolating cDNA clones, especially the original cDNA clone).

Hybridizing probes were made on the basis of the N-terminal amino acid sequence (main sequence) and amino acid sequences of tryptic peptides which had been obtained from highly purified TNF-BP. A cDNA which represents part of the cDNA coding for TNF-BP was obtained with the aid of these probes by means of PCR from the cDNA library HS913T. This cDNA has the following nucleotide sequence:

```
CAG GGG AAA TAT ATT CAC CCT CAA AAT AAT TCG ATT TGC TGT
ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA
GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC
TCC TTC ACA GCC TCA GAA AAC AAC AAG.
```

This DNA represents one of possible variants suited for hybridizing with TNF-BP-DNAs or TNF-BP-RNAs. (Such variants include, e.g., those DNA molecules that are obtained by PRC amplification with the aid of primers whose nucleotide sequence does not exactly correspond to the sequence searched for, e.g. because of restriction sites intended for cloning purposes or because of amino acids that have e.g. not definitely been determined in the amino acid sequence analysis). "TNF-BP-DNAs" and "TNF-BP-RNAs" are defined as being nucleic acids coding for TNF-BP or related proteins having the ability to bind TNF or nucleic acids which contain a sequence coding for such protein.

TNF-BP-DNAs (and TNF-BP-RNAs) also comprise cDNAs derived from mRNAs obtained by alternative splicing (or these mRNAs themselves). "Alternative splicing" means the removal of introns where different splice acceptor and/or splice donor sites are used from the same mRNA precursor. The resultant mRNAs differ from one another by the total or partial presence or absence of specific exon sequences, with a possible reading frame shift.

The cDNA (or variants thereof) which is first obtained according to the invention and contains part of the sequence coding for TNF-BP can thus be used as a hybridizing probe for obtaining TNF-BP-DNAs containing cDNA clones from cDNA libraries. Furthermore, it can be used as a hybridizing probe for mRNA preparations to isolate TNF-BP-RNAs and to produce therefrom e.g. enriched cDNA libraries permitting a considerably simplified and more efficient screening. Another application is the isolation of the desired DNAs from genomic DNA libraries with the aid of these DNAs as hybridizing probes.

The above-defined DNA (or its variants) is capable of hybridizing with DNAs (or RNAs) coding for TNF-BP or containing the sequence coding for TNF-BP. With the aid of this DNA as a probe it is also possible to obtain cDNAs coding for proteins, whose processing yields TNF-BP. Processing means the *in vivo* cleavage of partial sequences. As far as the N-terminus is concerned, this may be the signal sequence and/or other sequences and optionally, as far as the C-terminus is concerned, also

the transmembranic and cytoplasmatic region of the receptor. It is therefore possible with the aid of this hybridizing probe to screen suitable cDNA libraries for the presence of cDNA containing the complete sequence coding for a TNF receptor. (If required, this process can be performed in several steps).

According to the invention the cDNA of the above-defined sequence, which had been obtained by means of PCR from the cDNA library of the TNF- $\alpha$  induced fibrosarcoma cell line HS913 T (in  $\lambda$  gt11), was used for the repeated screening of the cDNA library, the lambda-DNA was prepared from the hybridizing clones, sucloned and sequenced. This led to a 1334 base-long cDNA insert containing the sequence coding for TNF-BP.

Hence, DNAs coding for a polypeptide with the ability to bind TNF or for a polypeptide of which this TNF binding protein represents a partial sequence were first provided for. This also covers DNAs coding for parts of such polypeptides.

The complete nucleotide sequence of the longest cDNA insert obtained is illustrated in Fig. 1.

This nucleotide sequence has a continuous open reading frame, starting with base 213, up to the end of the 1334 bp long cDNA inserts. Since in the same reading frame a stop codon is positioned 4 codons before the potential translation start codon ATG (213-215), it was assumed that the start codon is in fact the translation start codon used in vivo.

A comparison between the amino acid sequence derived from the nucleotide sequence and the amino acid sequences determined from the amino-terminal end of TNF-BP and tryptic peptides shows that there is a high degree of correspondence. As a result, the isolated cDNA contains the sequence coding for the authentic TNF-BP.

Starting from the N-terminus, the first sequence corresponding to a tryptic peptide cleavage sequence is the sequence of fraction 12 (Leu-Val-Pro-...), which had also been obtained as a side sequence in the analysis of the N-terminus of TNF-BP. This N-terminal leucine corresponds to the 30th amino acid in the cDNA sequence. Since the preceding segment of 29 amino acids is highly hydrophobic, and since TNF-BP is a secreted protein, the conclusion can be drawn that these 29 amino acids represent the signal peptide required for the secretion process, which is cleaved off during secretion (designated as S1-S29 in Fig. 1). The amino acid sequence obtained as the main sequence in the N-terminal analysis of TNF-BP corresponds to the amino acids beginning with Asp-12 in the cDNA sequence. This asparagine acid residue directly follows the alkaline dipeptide Lys-Arg. Since a very great number of proteins are proteolytically cleaved in vivo after this dipeptide, it can be assumed that TNF-BP with N-terminal Asp is not directly obtained by processing a precursor during the secretion process, but that the N-terminal 11 amino acids are cleaved off from the processed protein by extracellular proteases at a later time. The carboxy-terminal end of TNF-BP had been determined as Ile-Glu-Asn (C-terminal analysis; tryptic

peptide fraction 27: amino acids 159-172, tryptic peptide fraction 21: amino acids 165-172), with Asn corresponding to the position 172 in the cDNA sequence.

Potential N-glycosylation sites of the general formula Asn-Xaa-Ser/Thr, where Xaa may be any desired amino acid except for proline, are at the positions 25-27 (Asn-Asn-Ser), 116-118 (Asn-Cys-Ser) and 122-124 (Asn-Gly-Thr) of the TNF-BP cDNA sequence). (The assumption that Asn-25 is glycosylated follows from the fact that when the corresponding tryptic cleavage peptide was sequenced Asn could not be identified at this site).

The analysis of the nucleotide sequence and of the amino acid sequence derived therefrom shows in connection with the performed protein-chemical assays that TNF-BP is a glycosylated polypeptide having 172 amino acids, which is converted by proteolytic cleavage after the 11th amino acid into a glycoprotein with 161 amino acids. The following table shows the sequenced tryptic peptides and the corresponding amino acid sequences derived from the cDNA sequence:

| Fraction | Amino Acids |                                                                            |
|----------|-------------|----------------------------------------------------------------------------|
| -----    |             |                                                                            |
| 12       | 1- 8        |                                                                            |
| 1        | 12- 19      |                                                                            |
| 8        | 20- 32      |                                                                            |
| 14/I     | 36- 48      |                                                                            |
| 20       | 36- 53      |                                                                            |
| 11       | 54- 67      | (amino acids 66-67 had not<br>correctly been determined<br>on the peptide) |
| 14/II    | 79- 91      |                                                                            |
| 26       | 133-146     |                                                                            |
| 5        | 147-158     |                                                                            |
| 27       | 159-172     |                                                                            |

The resultant cDNA is the prerequisite for the production of recombinant TNF-BP.

As already mentioned, after the codon for Asn-172 the cDNA first isolated according to the invention does not contain the stop codon that would have to be expected by reason of the analysis of the C-terminus, but the open reading frame is continued. The region between Val-183 and Met-205 shows highly hydrophobic characteristics. This hydrophobic region of 22 amino acids, followed by a segment having a content of positively charged amino acids (Arg-206, Arg-209), exhibits the typical features of a transmembrane domain which anchors proteins in the cell membrane. By contrast, the protein portion following in the direction of the C-terminus is again highly hydrophilic.

The hydrophobicity profile is plotted in Fig. 2. (The hydrophobicity plot was made with the aid of the Mac Molly program (Soft Gene Berlin); the window width for the calculation of the values was 11 amino acids. Hydrophobic regions correspond to positive values, hydrophilic regions to negative values on the ordinate. The number of the amino acids, beginning with the start methionine S1, is shown on the abscissa).

As follows from the protein structure, the DNA coding for the soluble secreted TNF-BP is part of a DNA coding for a larger protein: This protein exhibits the features of a protein anchored in the cell membrane, contains TNF-BP in a manner typical of an extracellular domain and has a considerable segment typical of cytoplasmatic domains. Soluble TNF-BP is obviously obtained from this membrane-bound form by proteolytic cleavage just outside of the transmembrane domain.

The structure of the protein encoded from the obtained cDNA, together with the ability of TNF-BP to bind TNF, confirms the assumption that TNF-BP is part of a cellular surface receptor for TNF whose extracellular domain responsible for the binding of TNF can be cleaved off proteolytically and is found again in the form of the soluble TNF-BP. (This shall not rule out a situation in which with respect to the functional performance of the receptor said protein is possibly associated with one or several other proteins).

For the production of TNF-BP on a large scale it is advantageous not to start from the whole cDNA because attention would have to be paid to the requirement that TNF-BP has to be cleaved off from the part of the protein that represents the membrane-bound part of the TNF receptor. Rather, as already stated, a translation stop codon is expediently inserted after the codon for Asn-172 by directed mutagenesis to prevent any kind of protein synthesis from going beyond the C-terminal end of TNF-BP.

With the cDNA first obtained according to the invention, which represents a partial sequence of the DNA coding for a TNF receptor, it is possible to obtain the complete receptor sequence by amplifying the missing 3' end, for instance, by means of modified PCT (RACE = "rapid amplification of cDNA ends" (Frohman et al., 1988)) with the aid of a primer which was constructed on the basis of a sequence situated as much as possible towards the 3' end of the existing cDNA. An alternative method is the conventional screening of the cDNA library with the available cDNA or parts thereof as the probe.

According to the invention the rat-TNF-receptor cDNA was first isolated and, with a partial sequence therefrom, the complete human TNF receptor cDNA was obtained and expressed.

The subject matter of the present invention is a human TNF receptor and the DNA coding therefor. This definition comprises DNAs coding for forms which are C-terminal and/or N-terminal shortened, e.g. processed, or for modified forms (for example by changes at proteolytic

cleavage sites, glycosylation sites or certain regions of the domains) or for fragments, e.g. the various domains of the TNF receptor. In combination with the control sequences required for the expression, the DNAs can be used as part of recombinant DNA molecules, which are also the subject matter of the present invention, for the transformation of prokaryotic or eukaryotic host organisms. On the one hand, this paves the way for the production of the TNF receptor or modifications or fragments thereof in large quantities in a recombinant process, for instance, in order to be able to clarify the three-dimensional structure of the receptor. On the other hand, higher eukaryotic cells can be transformed with these DNAs to make studies on the mechanisms and dynamics of the TNF/receptor interaction, the signal transfer or on the relevance of the various receptor domains or segments thereof.

The recombinant TNF receptor (or fragments or modifications thereof) can be used for examining substances as to their interaction with TNF or the TNF receptor and their impact on the signal transfer induced by TNF. Such screenings (using proteins/fragments or correspondingly transformed higher eukaryotic cells) constitute the precondition for the identification of substances substituting TNF, inhibiting its binding to the receptor, or such substances that can block or intensify the mechanism of the signal transfer initiated by TNF.

One possibility of finding agonists and antagonists of TNF or the TNF receptor consists in establishing a high

capacity screening. A suitable cell line, preferably one that does not express an endogenous human TNF receptor, is here transformed with a vector which contains the DNA coding for a functional TNF receptor, which has possibly been modified in comparison with the natural sequence. The action of agonists and antagonists, respectively, can be examined in such a screening action by studying the response to the interaction of the substance with the receptor via a suitable reporter (changed enzyme activity, e.g. protein kinase C or gene activation, e.g. manganese superoxide dismutase, NF-KB).

Mechanisms and dynamics of the TNF/receptor interaction, the signal transfer and the corresponding role of the receptor domains can e.g. be studied by combining the DNA segments coding for the extracellular domain of the TNF receptor (or parts thereof) with DNA segments coding for various transmembrane domains and/or various cytoplasmatic domains and by expressing the same in eukaryotic cells. The resultant hybrid expression products might be suited for furnishing information on the respective relevance of the various receptor domains on the basis of possibly changed characteristics for the signal transduction, thereby facilitating purposeful screening.

The availability of the cDNA, or fragments thereof, coding for the TNF receptor is a precondition for obtaining the genomic DNA. To this end, a DNA library is screened under stringent conditions and the resultant clones are assayed as to whether they have the regulatory sequence elements required for the gene expression in

addition to the coding regions (e.g. checking as to the promotor function by fusion with coding regions of suitable reporter genes). Obtaining the genomic DNA sequence offers the possibility to examine the regulatory sequences located in the region not coding for the TNF receptor, especially in the 5'-flanking region, as to a possible interaction with known substances modulating the gene expression, e.g. transcription factors, steroids, and possibly to find new substances that might have a specific effect for the expression of this gene. The results of such studies offer the basis for the modulation of the TNF-receptor expression and thus for a direct purposeful use of such substances for the modulation of the TNF-receptor expression and thus for a direct influencing of the ability of the cell to interact with TNF. As a result, the specific reaction with the ligand and the resultant effects can be stopped.

The present invention also embraces DNAs which code for sub-types of the TNF receptor or its soluble forms which possibly have different characteristics as compared with the present TNF receptor. These are expression products which were obtained by way of alternative splicing and have changed structures in partial regions, for instance structures that can change the affinity and specificity for the ligand (TNF- $\alpha$ /TNF- $\beta$ ), or effect a change in the type and efficiency of the signal transfer.

With the help of the cDNA coding for the TNF receptor, it is possible to obtain nucleic acids which hybridize with the cDNA or segments thereof under conditions of low stringency and code for a polypeptide having the ability

to bind TNF, or contain the sequence coding for such polypeptide.

Under a further aspect the subject matter of the present invention is the recombinant TNF-BP, preferably in secretable form, which represents the soluble part of the TNF receptor of the invention or the DNA coding therefor.

TNF-BP which is secreted into the cell supernatant can be produced by incorporating a DNA construct containing the sequence coding for TNF-BP with a sequence coding for a signal peptide under control of a suitable promotor in suitable host organisms, especially in eukaryotic, preferably higher eukaryotic cells.

When a signal peptide is used with respect to the secretion of the protein, the DNA coding for the signal peptide is expediently inserted before the codon for Asp-12 to obtain a uniform product. As a rule, any signal peptide is suited that guarantees the secretion of the mature protein in the corresponding host organism. Optionally the signal sequence can also be placed before the triplet coding for the Leu-1, and in such a case it might be necessary to separate the TNF-BP form created at the N-terminus by cleavage of the peptide consisting of 11 amino acids, from the unprocessed or not completely processed TNF-BP in an additional purification step.

Since the cDNA after the codon for Asn-172, which represents the C-terminus because of the C-terminal analysis, does not contain a stop codon, a translation stop codon is expediently incorporated after the codon

for Ans 172 by directed mutagenesis with respect to the expression of TNF-BP.

The DNA coding for TNF-BP may be modified by mutation, transposition, deletion, addition or shortening, provided that such modified DNAs code for (poly)peptides having the ability to bind TNF. Such modifications may be changes of one or more of the potential glycosylation sites, which may perhaps not be required for biological activity, by replacing e.g. the Asn codon by a triplet coding for another amino acid. Taking into account that the biological activity is retained, modifications may be performed, which result in changes of the disulfide bridges (for example reducing the number thereof).

Hence, the stated DNA molecules are a precondition for the construction of recombinant DNA molecules, which are also the subject matter of the present invention. Suitable host organisms can be transformed and grown and the protein can be obtained with such recombinant DNA molecules in the form of expression vectors containing the DNA coding for a protein with TNF-BP activity and optionally modified in a suitable manner, preferably with a preceding signal sequence, and the control sequences required for the expression of the protein.

Like with possible modifications of the DNA sequence, host organisms suited for the expression are selected, particularly with respect to the biological activity of the protein to bind TNF. Moreover, the standard criteria applicable in the production of recombinant proteins, e.g. compatibility with the selected vector, processing

capacity, isolation of the protein, expression characteristics, safety and cost aspects play also a role in the decision on the host organism. The selection of a suitable vector also follows from the host intended for the transformation. As a rule, all vectors are suited that replicate and express the DNAs coding for TNF-BP according to the invention (or modifications thereof).

With respect to the biological activity of the protein, it is especially the possible relevance of the criteria determined in the natural protein, namely glycosylation and high amount of cysteine residues, that must be paid attention to as to the property to bind TNF as far as the expression of the TNF-BP coding DNA is concerned. Therefore, eukaryotes, especially suitable expression systems of higher eukaryotes, are used for the expression.

Both transient expression and permanent expressions of TNF-BP were detected in eukaryotic cells within the scope of the present invention.

The recombinant TNF-BP of the invention, as well as suitable modifications thereof which have the ability to bind TNF can be used in the prophylactic and therapeutic treatment of the human or animal body in the case of indications where a detrimental effect of TNF- $\alpha$  takes place.

Since a TNF- $\beta$  inhibiting effect was also detected in TNF-BP, it (or the related or modified polypeptides) can be used in suitable dosage, optionally in a form modified

with respect to an enhanced affinity to TNF- $\beta$ , also for inhibiting the effect of TNF- $\beta$  in the organism.

Furthermore, the subject matter of the present invention are therefore pharmaceutical preparations containing an amount of recombinant TNF-BP effectively inhibiting the biological activity of TNF- $\alpha$  and/or TNF- $\beta$ , or a related polypeptide with the ability to bind TNF.

Pharmaceutical preparations are especially suited for parenteral application in the case of the above-mentioned indications where a detrimental effect of TNF is observed, e.g. in the form of lyophilisates or solutions. These contain TNF-BP or a therapeutically effective functional derivative in a therapeutically effective amount, possibly together with physiologically compatible additives, such as stabilizers, buffers, preservatives.

The dosage depends above all on the respective indication and the specific form of administration, for instance whether performed locally or systemically. The quantity of the single doses optionally depends on the individual assessment of the case, taking into account factors such as general state, case history, age, weight, sex of the patient, etc. Of importance to the determination of the therapeutically effective dose is the consideration of the amount of released TNF responsible for the disease and the amount of endogenous TNF-BP. As a rule, it can be assumed that at least the same molar amount of TNF-BP, possibly a multiple surplus, is needed for the released amount of TNF for effectively treating a disease triggered by TNF.

The above problem was solved in detail as follows:

As for the highly purified TNF-BP, the N-terminal amino acid sequence and the amino acid sequences of peptides obtained by tryptic digestion of the protein were determined.

Furthermore, the C-terminus was determined by carboxypeptidase P, digestion, derivatization of the cleaved amino acids and chromatographic separation.

From the peptide sequences obtained by tryptic digestion, and with respect to the use thereof in the PCR for the preparation of oligonucleotides, regions were selected, on the one hand, from the N-terminus and, on the other hand, from a tryptic peptide in such a manner that the complexity of mixed oligonucleotides is as low as possible as for hybridization with cDNA. Based on these two regions, a respective set of mixed oligonucleotides were prepared, the one derived from the region at the N-terminus being synthesized in accordance with the mRNA and the one derived from the tryptic peptide being synthesized in a reversely complementary manner as to the mRNA. To facilitate subsequent cloning of a PCR amplified segment, the set of oligonucleotides derived from the tryptic peptide was provided with a BamHI restriction site.  $\lambda$  DNA was then isolated from the TNF- $\alpha$  induced fibrosarcoma cDNA library and a TNF-BP sequence was amplified therefrom by means of PCR. The resultant fragment was cloned and sequenced; it had 158 nucleotides and contained the sequence coding for the tryptic peptide

20 between the two sequence segments derived from the primer oligonucleotides.

This DNA fragment was subsequently radioactively labeled and used as a probe for isolating cDNA clones from the fibrosarcoma library. The procedure was such that plaques were first hybridized with the probe, phages of hybridized plaques were isolated and  $\lambda$  DNA was obtained therefrom. Individual cDNA clones were subcloned and sequenced; two of the characterized clones contained the sequence coding for TNF-BP.

This sequence forms part of the sequence coding for a TNF receptor.

After shortening the 5'-noncoding region and introducing a stop codon after the codon for the C-terminal amino acid of the natural TNF-BP, the cDNA was inserted into a suitable expression plasmid, so that eukaryotic cells were transformed and the expression of TNF-BP was detected by means of ELISA.

The still unknown 3' region of the TNF receptor was found by screening a rat brain cDNA library from the rat glia tumor cell line C6 with a TNF-BP probe and by isolating the complete cDNA coding for the rat TNF receptor.

The 3' located fragment of this cDNA, of which it was assumed that it corresponds to the missing 3' region behind the EcoRI site of the human TNF receptor, was used as a probe for screening the HS913T cDNA library once

again. This yielded a clone containing the whole DNA coding for the TNF receptor.

After shortening of the 5' non-coding region the cDNA was inserted into an expression plasmid and the expression of human TNF receptor in eukaryotic cells was detected via the binding of radioactively labeled TNF.

It was confirmed by means of the Northern blot analysis that the isolated cDNA almost corresponds to the entire TNF-R mRNA (the slight discrepancy is due to the absence of part of the 5'-noncoding region). The conclusion can be drawn that the expressed protein is the complete TNF receptor.

The invention is illustrated with reference to the following preliminary tests and examples:

#### Preliminary Test 1

##### Preparation of highly purified TNF-BP

##### a) Concentration of urine

200 l of dialysis urine of uremia patients, stored in bottles containing EDTA (10 g/l), Tris (6 g/l),  $\text{NaH}_3$  (1 g/l) and benzamidine hydrochloride (1 g/l) and stored at a cool place, were concentrated by ultrafiltration by means of a highly permeable hemocapillary filter with an asymmetrical hollow fiber membrane (FH 88H, Gambro) to 4.2 l with a protein content of 567 g. The concentrated urine was dialyzed against 10mM/l Tris HCl, pH 8. During

this process, and like in the subsequent steps (except for reversed-phase chromatography), 1mM/1 benzamidine hydrochloride was added to counteract proteolytic digestions. All of the subsequent purification steps were performed at 4°C, unless stated otherwise.

#### b) Ion Exchange Chromatography

This step was performed by charging DEAE Sephacel columns (2.5 x 40 cm) with samples of concentrated and dialyzed urine containing about 75 g protein each. Elution was carried out with 800 ml of an NaCl /10mM Tris/HCL pH-8 gradient, the NaCl concentration amounting to 0 to 0.4 M. The fractions of seven columns containing the TNF-BP, having a total protein content of 114 g, were stored at -20°C.

#### c) Affinity Chromatography

To prepare the TNF Sepharose column, rTNF- $\alpha$  (15 mg) in 0.1 M NaHCO<sub>3</sub>, 1 M NaCl, pH 9 (coupling buffer) was coupled to 1.5 g cyanogenic bromide-activated Sepharose 4B (Pharmacia). The Sepharose was swollen in 1mM HCl and washed with coupling buffer. After addition of rTNF- $\alpha$  the suspension was rotated at room temperature for 2 hours. The excess of CNBr groups was blocked by rotation for one and a half hours with 1M ethanolamine, pH 8. The TNF Sepharose was alternately washed several times in 1M NaCl, 0.1 M sodium acetate pH 8 and 1 M NaCl, 0.1 M boric acid pH 4 and subsequently stored in phosphate-buffered saline with 1 mM benzamidine hydrochloride. The fractions obtained from step b) were set to a concentration of 0.2

M NaCl, 10mM Tris/HCl, pH 8. The TNF Sepharose was packed into a column and washed with 0.2 M NaCl, 10mM Tris HCl, pH 8, and the TNF-BP containing fractions corresponding to about 30 g protein, were applied at a flow rate of 10 ml/h and profusely washed with 0.2 M NaCl, 10mM Tris HCl, pH 8 until an absorption could no longer be detected in the eluate at 280nm. TNF-BP was then eluted with 0.2 M glycine/HCl, pH 2.5.

TNF-BP containing fractions of 4 separations were combined and lyophilized up to an end concentration of 10 mg/ml after addition of polyethyleneglycol (MG 6000). The lyophilized sample was dissolved in distilled water and dialyzed against distilled water. (The dialyzed sample (4 ml) was stored in a deeply frozen state.)

This purification step led to a further enrichment by about 9000 times in comparison with the preceding step. SD-PAGE (performed as described in preliminary test 2) of the TNF-BP containing fractions showed the elution of three main components with molecular weights of 28,000, 30,000 and 50,000.

#### d) Reversed Phase Chromatography

An aliquot amount (1 ml) of the fractions obtained from step c) with an addition of 0.1% trifluoroacetic acid was applied to a ProRPC HR 5/10 column (Pharmacia) connected to an FPLC system (Pharmacia). The column was equilibrated with 0.1% trifluoroacetic acid and charged at room temperature with a linear 15 ml gradient of 10 volume percent to 50 volume percent of acetonitrile

containing 0.1% trifluoroacetic acid; the flow rate was 0.3 ml/min. Fractions of 0.5 ml were collected and the absorption at 280 nm as well as the activity of the TNF- $\alpha$  binding protein were determined with the aid of the competition binding assay, as indicated in Example 1, with 0.01  $\mu$ l sample being respectively used. TNF-BP eluted as a single activity peak according to a sharp UV absorption peak.

This last purification step yielded an increase in specific activity by about 29 times; the total increase in activity as compared with the starting material (concentrated dialysis urine) amounted to about  $1.1 \times 10^6$  times.

SDS-PAGE of the reduced and non-reduced sample, carried out as indicated in preliminary test 2, yielded a diffuse band which pointed to the presence of a single polypeptide with a molecular weight of about 30,000. The diffuse appearance of the bands might be due to the presence of one or more heterogenous glycosylations and/or a second polypeptide present in a small amount. The assumption that this might be a polypeptide having the N-terminus determined as a side sequence in preliminary test 3d, which polypeptide is extended at the N-terminus as compared with TNF-BP, was confirmed by the sequence of the cDNA according to which a segment of 11 amino acids is present between the signal sequence and Asn (pos. 12), the sequence of the segment corresponding to the N-terminal side sequence and the segment being obviously cleaved off from the processed protein.

## Preliminary Test 2

## SDS Polacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method of Laemmli (Laemmli 1970) on flat gels having a length of 18 cm, a width of 16 cm and a thickness of 1.5 mm and 10 pockets by means of an LKB 2001 electrophoresis unit. The protein content of the samples from purification steps c) and d) (preliminary test 1) was determined by means of Bio-Rad Protein Assay and calculated from the absorption at 280 nm, with a content of 1 mg TNF-BP/ml being assigned to an absorption of 1.0.

The samples containing about 25  $\mu$ m protein (from preliminary test 1c) and about 5  $\mu$ g (from 1d) in reduced ( $\beta$ -mercaptoethanol) and non-reduced form were applied to a 3% concentration gel and a 5% to 20% linear polyacrylamide gradient gel. Electrophoresis was performed at 25mA/gel without cooling. Phosphorylase B (MW 94,000), bovine serum albumin (MW 67,000), ovalbumin (MW 43,000), carboanhydrase (MW 30,000), soybean trypsin inhibitor (MW 20,100) and  $\alpha$ -laktalbumin (MW 14,400) were used as molecular weight markers (Pharmacia). The gels were stained with Coomassie blue in 7% acetic acid/40% ethanol and destained in 7% acetic acid/25% ethanol.

The result of the SDS-PAGE showed TNF-BP as a polypeptide chain with a molecular weight of about 30,000.

## Preliminary Test 3

#### a) Sample Preparation

15  $\mu$ g of the protein purified according to the preliminary test 1d) were desalted via reversed phase HPLC and further purified. To this end a Bakerbond WP C18 column (Baker; 4.6 x 250 mm) and 0.1% trifluoroacetic acid were used in water (eluent A) and in acetonitrile (eluent B) as the mobile phase. The increase in gradient was 20% to 68% of eluent B in 24 min. Detection was performed at 214 nm and at 280 nm at the same time. The TNF-BP containing fraction was collected, dried and dissolved in 75  $\mu$ l 70% formic acid and directly used for the amino acid sequence analysis.

#### b) Amino Acid Sequence Analysis

The automatic amino acid sequence analysis was performed with an Applied Biosystems 477 A Liquid Phase Sequenator by on-line determination of the released phenylthiohydantoin derivatives by means of an Applied Biosystems Analyzer, model 120 A PTH. It showed the following N-terminal sequence as the main sequence: (about 80% of the protein amount): Asp-Ser-Val-Xaa-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-.

Apart from this, the following side sequence could be detected: Leu-(Val)-(Pro)-(His)-Leu-Gly-Xaa-Arg-Glu-. (The amino acids put into brackets could not be clearly identified.)

## Preliminary Test 4

## SDS-PAGE

The sample preparation was performed in the same manner as in the preliminary test 3, except for the difference that the sample amount was 10  $\mu\text{g}$ . The sample was received in 50  $\mu\text{l}$  water and divided into 4 portions. One of the four aliquot parts was reduced by means SDS-PAGE according to the method of Laemmli (24) with DTT (dithiothreitol) for determining the purity and was separated on minigels (Höfer, 55x80x0.75mm, 15%); the marker indicated in preliminary test 8 was used as the molecular weight marker. Staining was performed according to the Oakley method (Oakley, et al., 1986). The electrophoretogram is illustrated in Fig. 9. It shows a single band at a molecular weight of about 30,000.

## Example 1

## a) Tryptic Peptide Mapping

About 60  $\mu\text{g}$  of the protein purified according to preliminary test 1d) were desalted via reversed phase HPLC and thus further purified. To this end a Bakerbond WP C18 column (Baker; 4.6 x 250 mm) and 0.1% trifluoroacetic acid were used in water (eluent A) and in acetonitrile (eluent B) as the mobile phase. The gradient increase was 20% to 68% of eluent B in 24 min. Detection was in parallel at 214 nm and at 280 nm. The TNF-BP containing fraction (retention time: about 13.0

min) was collected, dried and dissolved in 60  $\mu$ l 1% ammonium bicarbonate.

1% w/w, corresponding to 0.6  $\mu$ g trypsin (Boehringer Mannheim), was added to this solution and the reaction mixture was incubated at 37°C for 6 hours. 1% w/w trypsin was subsequently added once again and incubation was continued overnight.

For the reduction of the disulfide bridges the reaction batch had then added thereto 60  $\mu$ l 6 M urea and 12  $\mu$ l of 0.5 M dithiothreitol and was allowed to stand at room temperature for 2 hours.

The resultant tryptic cleavage peptides were separated via reversed phase HPLC, a Delta PAK C18 column (Waters, 3.9 x 150 mm, 5  $\mu$ m particle diameter, 100 Å pore diameter) being used at 30°C and 0.1% trifluoroacetic acid in water (eluent A) and in acetonitrile (eluent B) as the mobile phase. The gradient increase was 0 to 55% of eluent B within 55 min; 55% B was then maintained for 15 min. The flow rate was 1 ml/min; detection was in parallel at 214 nm (0.5 AUFS) and at 280 nm (0.05 AUFS).

#### b) Sequence Analysis of Tryptic Peptides

Some of the tryptic cleavage peptides of TNF-BP obtained according to a) were subjected to automatic amino acid sequence analysis. To this end the corresponding fractions from reversed phase HPLC were collected, dried and dissolved in 75  $\mu$ l 70% formic acid. These solutions were directly used for sequencing in an Applied

Biosystems 477 A Pulsed Liquid Phase Sequenator. Table 1 shows the results of the sequence analysis of the tryptic peptides; the amino acids put into brackets could not be identified for sure. The indication "Xaa" means that the amino acid could not be identified at this position.

The amino acid at position 6 could not be identified in fraction 8. The sequence -Xaa-Asn-Ser- for the position 6-8 presumably indicates that the amino acid 6 is present in glycosylated form.

In fraction 17 the amino acid in position 6 could also not be identified. The sequence -Xaa-Asn-Ser (already appearing in fraction 8) for the positions 6 to 8 might indicate that the amino acid 6 is present in glycosylated form. The first 13 amino acids of fraction 17 are largely identical with fraction 8; thus fraction 17 is probably a peptide obtained by incomplete tryptic cleavage.

The identity of fraction 21 with the positions 7 to 14 of fraction 27 is striking. Both in fraction 21 and in fraction 27 the sequence is suddenly interrupted after the amino acid asparagine (positions 8 and 14) although a tryptic cleavage has not to be expected here. This might indicate that the amino acid asparagine (position 8 in fraction 21 and position 14 in fraction 27) could be the C-terminal amino acid of TNF-BP.

The large identity between the sequence of fraction 12 appearing only in a small amount and the side sequence of the N-terminus determined in the preliminary test 10 is striking. The fact that the proteins of the main and side sequences could not be separated on an analytical reversed-phase HPLC column (preliminary test 3b)

indicated that the protein with the side sequence is a form of TNF-BP extended at the N-terminus, which is converted by processing substantially into the protein with the main sequence.

Fraction Amino Acid Sequence

|       |                                                                                               |
|-------|-----------------------------------------------------------------------------------------------|
| 1     | Asp - Ser - Val - Cys - Pro - Gln - Gly - Lys                                                 |
| 2     | Xaa - Xaa - Leu - Ser -(Cys)- Ser - Lys                                                       |
| 3     | Asp - Thr - Val - (Cys)- Gly -(Cys)- Arg                                                      |
| 4     | Glu - Asn - Glu - (Cys)- Val - Ser - (Cys) -<br>Ser - Asn -(Cys) - Lys                        |
| 5     | Glu - Asn - Glu -(Cys)- Val - Ser - (Cys)-<br>(Ser)- Asn - (Cys)- Lys - (Lys)                 |
| 8     | Tyr - Ile - His - Pro - Gln - Xaa - Asn - Ser<br>- Ile - Xaa - Xaa - Xaa - Lys                |
| 11    | Glu - Cys - Glu - Ser - Gly - Ser - Phe - Thr<br>- Ala - Ser - Glu - Asn -(Asn) - (Lys)       |
| 12    | Leu - Val - Pro - His - Leu - Gly - Asp - Arg                                                 |
| 13    | Lys - Glu - Met - Gly - Gln - Val - Glu - Ile<br>- Ser - Ser - (Cys)- Thr - Val - Asp - (Arg) |
| 14/I  | Gly - Thr - Tyr - Leu - Tyr - Asn - Asp - Cys<br>- Pro - Gly - Pro - Gly - Gln -              |
| 14/II | (Glu) - Met - Gly - Gln - Val -(Glu)- (Ile) -<br>(Ser)- Xaa - Xaa - Xaa - (Val) -(Asp)-       |

- 15        Lys - Glu - Met - Gly - Gln - Val - Glu - Ile  
           - Ser - Ser - (Cys) - Thr - Val - Asp - Arg -  
           Asp - Thr - Val - (Cys) - Gly -
- 17        Tyr - Ile - His - Pro - Gln - Xaa - Asn - Ser  
           - Ile - (Cys) - (Cys)- Thr - Lys - (Cys) - His  
           - Lys - Gly - Xaa - Tyr -
- 20        Gly - Thr - Tyr - Leu - Tyr - Asn - Asp - Cys  
           - Pro - Gly - Pro - Gly - Gln - Asp - Thr -  
           Xaa - Xaa - Arg
- 21        Leu - (Cys) - Leu - Pro - Gln - Ile - Glu -  
           Asn
- 26        Gln - Asn - Thr - Val -(Cys)- Thr - Xaa -  
           (His)- Ala - Gly - Phe - (Phe) - Leu - (Arg)
- 27        Ser - Leu - Glu - (Cys) - Thr - Lys - Leu -  
           (Cys)- Leu - Pro - Gln - Ile - Glu - Asn

Table 1:        Amino acid sequences of the analyzed  
 tryptic peptides of TNF-BP

#### Example 2

#### Analysis of the C-terminus

This analysis was performed in accordance with the principle of the method described in (Hsieng et al., 1988).

About 60  $\mu$ g of the protein purified according to preliminary test 2d) were desalted via reversed phase HPLC and thus further purified. To this end a Bakerbond WP C18 column (Baker; 4.6 x 250 mm) and 0.1% trifluoroacetic acid in water (eluent A) and in acetonitrile (eluent B) were used as the mobile phase. Gradient increase was 20% to 68% of eluent B in 24 min. Detection was in parallel at 214 nm and at 280 nm. The TNF-BP containing fraction (retention time about 13.0 min) was collected, dried and dissolved in 120  $\mu$ l 10 mM sodium acetate (set to pH 4 with 1 N HCl).

6  $\mu$ l Brij 35 (10 mg/ml in water) and 1.5 carboxypeptidase P (0.1 mg/ml in water, Boehringer Mannheim, No. 810142) were added to this solution. This corresponds to a weight ratio of enzyme to protein of 1 to 400 (Frohman et al., 1988).

A sample of 20  $\mu$ l was taken from the reaction mixture immediately after addition of the enzyme and the enzymatic reaction was interrupted by acidification with 2  $\mu$ l of concentrated trifluoroacetic acid and by freezing at  $-20^{\circ}\text{C}$ .

The reaction mixture was allowed to stand in the refrigerator (about  $8^{\circ}\text{C}$ ), and samples of 20  $\mu$ l each were taken after 10, 20, 60 and 120 minutes. The rest of the reaction mixture was left at room temperature for another 120 minutes. Immediately after removal all of the samples were acidified by addition of 2  $\mu$ l of concentrated

trifluoroacetic acid and frozen at  $-20^{\circ}\text{C}$ , whereby the enzymatic reaction was interrupted.

In parallel with the described sample batch with about 60  $\mu\text{g}$  TNF-BP, a blank reagent value, which had no protein added thereto, was used under identical conditions.

After the last sampling all samples were dried in a Speed Vac Concentrator for 30 minutes, mixed with 10  $\mu\text{l}$  of a solution consisting of 2 parts of ethanol, 2 parts of water and 1 part of triethylamine (= "Redrying Solution" of the Picotag Amino Acid Analysis System of Waters) and once again dried for a short period. The samples were then mixed with 20  $\mu\text{l}$  each of the "Derivatization Reagent" (7:1:1:1 = ethanol : water : triethylamine : phenylisothiocyanate; Picotag System) for the derivatization of the amino acids cleaved off from the C-terminus, allowed to stand at room temperature for 20 minutes and then dried in a Speed Vac Concentrator for 1 hour.

The samples were dissolved in 100  $\mu\text{l}$  "Sample Diluent" (Picotag System of Waters) for analyzing the derivatized amino acids. Of these solutions 50  $\mu\text{l}$  each were analyzed with the aid of reversed phase HPLC (column, mobile phase and gradient according to the original instructions of the Picotag system of Waters). The chromatograms of the samples and the blank reagent values were compared with the chromatogram of an analogously derivatized mixture (100 pmol/amino acid) of standard amino acids (Beckman).

As becomes apparent from the quantitative results of the Picotag amino acid analysis (Table 2), it is very likely that asparagine is the C-terminal amino acid of TNF-BP. Apart from asparagine, glutamic acid and a small amount of isoleucine could be detected after a reaction time of 240 minutes. Amounts of other amino acids significantly lying above the blank reagent value could not be found even after a reaction time of 240 minutes. This result (-Ile-Glu-Asn as the C-terminus) supports the assumption derived from the N-terminal sequencing of the tryptic peptides 21 and 27 that the amino acids -Ile-Glu-Asn (Example 1b) identified at the C-terminus in these peptides represent the C-terminus of TNF-BP.

| Reaction time |            | Integration units for the amino acids |            |  |
|---------------|------------|---------------------------------------|------------|--|
|               | Isoleucine | Glutamic Acid                         | Asparagine |  |
| -----         |            |                                       |            |  |
| 0             | -          | -                                     | -          |  |
| 10            | -          | -                                     | -          |  |
| 20            | -          | -                                     | 83,304     |  |
| 60            | -          | -                                     | 168,250    |  |
| 120           | -          | -                                     | 319,470    |  |
| 240           | 85,537     | 152,350                               | 416,570    |  |
| -----         |            |                                       |            |  |

Table 2: Quantitative evaluation of the Picotag amino acid analysis after reaction of carboxypeptidase P with TNF-BP.

Methods regarding Examples 3 to 7:

Unless explicitly stated otherwise, molecular-biological standard methods which can be looked up in relevant reference books or comply with the conditions recommended by manufacturers were used in the following examples. To simplify the description of the following examples, methods or designations that will often recur shall be described briefly hereinafter:

"Cutting" or "digesting" of DNA refers to the catalytic cleavage of DNA by means of restriction endonucleases (restriction enzymes) at sites (restriction sites) specific thereof. Restriction endonucleases are commercially available and are used under conditions recommended by manufacturers (buffer, bovine serum albumin (BSA) as carrier protein, dithiothreitol (DTT) as protection against oxidation). Restriction endonucleases are normally designated by a capital letter, most of the time followed by small letters and normally a Roman numeral. The letters depend on the microorganism from which the corresponding restriction endonuclease was isolated (for instance: Sma I: *Serratia marcescens*). About 1  $\mu$ g DNA is normally cut with one or several units of the enzyme in about 20  $\mu$ l buffer solution. Normally, there is an incubation period of 1 hour at 37°C, but it may be varied in accordance with the manufacturer's instructions of use. After the cutting operation the 5' phosphate group is sometimes removed by incubation with alkaline phosphatase of calf intestine (CIP). This helps to prevent an undesired reaction of the specific site in a subsequent ligase reaction (e.g. circularization of a

linearized plasmid without insertion of a second DNA fragment). Unless stated otherwise, DNA fragments are normally not dephosphorylated after cutting with restriction endonucleases. Reaction conditions for the incubation with alkaline phosphatase can e.g. be gathered from the M13 Cloning and Sequencing Handbook (Amersham, PI/129/83/12). After incubation protein is removed by extraction with phenol and chloroform and the DNA is precipitated from the aqueous phase by addition of ethanol.

"Isolating" a specific DNA fragment means the separation of the DNA fragments obtained by restriction digestion, e.g. on a 1% agarose gel. After electrophoresis and visualization of the DNA in UV light by staining with ethidium bromide (EtBr) the desired fragment is localized with the help of the molecular weight marker, which has also been applied, and is bound by further electrophoresis to DE 81 paper (Schleicher and Schüll). The DNA is washed by rinsing with low-salt buffer (200 mM NaCl, 20 mM Tris pH = 7.5, 1 mM EDTA) and subsequently eluted with a high-salt buffer (1 M NaCl, 20 mM tris pH = 7.5, 1 mM EDTA). The DNA is precipitated by addition of ethanol.

"Transformation" means the incorporation of DNA into an organism, so that the DNA can be replicated there, integrated either extrachromosomally or chromosomally. Transformation of E.coli is in line with the method indicated in the M13 Cloning and Sequencing Handbook (Amersham, PI/129/83/12).

"Sequencing" of a DNA means the determination of the nucleotide sequence. To this end the DNA to be sequenced is first cut with different restriction enzymes, and the fragments are incorporated into correspondingly cut M13 mp8, mp9, mp18 or mp19 double-strand DNA, or the DNA is fragmented by means of ultrasonics, the ends are repaired and the size-selected fragments are incorporated into Sma I cut, dephosphorylated M13 mp8 DNA (Shotgun Method). After transformation of E.coli JM 101 single-strand DNA is isolated from recombinant M13 phages according to the M13 Cloning and Sequencing Handbook (Amersham, PI/129/83/132) and sequenced according to the dideoxy method (Sanger et al., 1977). An alternative to the use of the Klenow fragment of the E.coli DNA polymerase I is the T7-DNA polymerase ("Sequenase", United States Biochemical Corporation). The sequence reactions are carried out in accordance with the manual "Sequenase: Step-by-Step Protocols for DNA Sequencing with Sequenase" (Version 2.0).

Another sequencing method is the cloning of the DNA to be sequenced in a vector which, inter alia, carries a replication origin of a DNA single-strand phage (M13, f1) (e.g. Bluescribe or Bluescript M13 of Stratagene). After transformation of E. coli JM101 with the recombinant molecule the transformants can be infected with a helper phage, e.g. M13K07 or R408 of Promega). As a result, a mixture of helper phages and packed, single-stranded recombinant vector is obtained. The sequencing template is processed by analogy with the M13 method. Double-stranded plasmid DNA was denatured and directly sequenced

in accordance with the above-mentioned sequencing manual by alkali treatment.

The sequences were evaluated by means of computer programs originally developed by R. Staden (Staden et al., 1982) and modified by CH. Pieler (Pieler, 1987).

"Ligating" means the process of forming phosphodiester bonds between two end of double-stranded DNA fragments. Between 0.02 and 0.2  $\mu\text{g}$  DNA fragments in 10  $\mu\text{l}$  are normally ligated with about 5 units T4-DNA ligase in a suitable buffer solution (Maniatis et al., 1982).

"Preparation" of DNA from transformants means the isolation of the plasmid DNA from bacteria by means of the alkaline SDS method, modified according to Birnboim and Doly, under omission of the lysozyme. The bacteria from 1.5 to 50 ml culture are used.

"Oligonucleotides" are short polydeoxynucleotides which are chemically synthesized. The Applied Biosystems Synthesizer Model 381A was used therefor. The oligonucleotides are processed in compliance with the Model 381A User Manual (Applied Biosystems). Sequence primers are directly used without further purification. Other oligonucleotides are purified up to a chain length of 70 by the "OPC" method (OPC = oligonucleotide purification column, Applied Biosystems, Product Bulletin, January 1988). Longer oligonucleotides are purified by polyacrylamide gel electrophoresis (6% acrylamide, 0.15% bisacrylamide, 6 M urea, TBE buffer) and desalted after elution from the gel via a G-25 Sepharose column.

## Example 3

## Preparation of TNF-BP Specific Hybridizing Probes

The selection of the oligonucleotides was made with respect to the use thereof for the amplification of cDNA by means of PCR:

- a) A heptapeptide region which permits the lowest complexity of a mixed oligonucleotide for hybridization on cDNA was selected from the N-terminal amino acid sequence of the TNF binding protein (main sequence obtained from preliminary test 3 and Example 1, fraction 1)

Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-.

These are the amino acids 6 to 12. To reduce the complexity of the mixed oligonucleotide, four mixed oligonucleotides were produced with a complexity of 48 each. The oligonucleotides were prepared in the direction of the mRNA; they are thus oriented towards the 3' end of the sequence and are identical with the noncoding strand of the TNF-BP gene:

Gln-Gly-Lys-Tyr-Ile-His-Pro

5'CAA GGT AAA TAT ATT CAT CC 3'TNF-BP #3/1 EBI-1639

G G C C C

A

5'CAA GGC AAA TAT ATT CAT CC 3'TNF-BP #3/2 EBI-1640

G G C C C

A

5'CAA GGA AAA TAT ATT CAT CC 3'TNF-BP #3/3 EBI-1641

G G C C C

A

5'CAA GGG AAA TAT ATT CAT CC 3'TNF-BP #3/4 EBI-1642

G G C C C

A

- b) A peptide region was selected from the amino acid sequence of a tryptic peptide (fraction 11 of the tryptic digest) of the amino acid sequence

Glu-Cys-Ser-Gly-Ser-Phe-Thr-Ala-Ser-(Glu/Cys)-Asn-Asn-Lys  
(cf. Example 1),

and another set of mixed oligonucleotides were  
synthetized:

-Phe-Thr-Ala-Ser-Glu-Asn-Asn-Lys

Cys

TNF-BP #4/5 (EBI-1653):

3'AAA TGA CGG AGA CTC TTG TTG TT CCTAGGG 5'

G G T T T

T

TNF-BP #4/6 (EBI-1654):

3'AAA TGA CGG TCA CTC TTG TTG TT CCTAGGG 5'

G G T G T

T

TNF-BP #4/7 (EBI-1657):

3'AAA TGA CGG AGA ACA TTG TTG TT CCTAGGG 5'

G G T T G

T

TNF-BP #4/8 (EBI-1658):

3'AAA TGA CGG TCA ACA TTG TTG TT CCTAGGG 5'

G G T G G

T

The oligonucleotides were synthesized complementary to the mRNA and thus oriented towards the 5' end of the sequence. To be able to efficiently clone the amplified DNA fragment following the PCR, a BamHI linker was also provided at the 5' end of the oligonucleotides. For instance, if the oligonucleotides TNF-BP #4/5-8 are jointly used with TNF-BP #3/1-4 for the PCR on the whole  $\lambda$ -DNA of a library, a possibly resulting DNA fragment can be further cut with BamHI. The partner oligonucleotides yield a straight end at the 5' terminus, the fragment can thus be cloned into the SmaI-BamHI sites of a suitable vector.

Each mixed oligonucleotide TNF-BP #4/5-8 is a mixture of 48 individual nucleotides and does not take into account a few codons, namely:

|     |             |
|-----|-------------|
| Thr | ACG         |
| Ala | GCG and GCT |
| Ser | TCG and TCC |
| Asn | AAT.        |

As far as GCT is concerned, the possibility is taken into account that the triplet CGG which is complementary to GCC (Ala) may be effective by formation of a G-T bridge; as for TCG (Ser) and AAT (Asn), the same observation holds true for AGT and TTG, respectively.

ACG, GCG and TCG are very rare codons (CG rule) and were therefore not taken into account.

## Example 4

Amplification of a partial sequence coding for TNF-BP from a cDNA library

a) Isolation of  $\lambda$ -DNA from a cDNA library

The cDNA library was made according to the method described in EP-A1-0293 657 for human placental cDNA library, except for the difference that  $10^9$  fibrosarcoma cells of the cell line HS 913 T which had been cultivated under stimulation with human TNF- $\alpha$  (10 ng/ml) were used as starting material. Instead of  $\lambda$  gt10  $\lambda$ , gt11 was used (cDNA synthesis: Amersham RPN 1256; EcoRI digested  $\lambda$  gt11 arms: Promega Biotech; in vitro packing of the ligated DNA: Gigapack Plus, Stratagene).

5 ml of the phage supernatant of the amplified cDNA library of the human fibrosarcoma cell line HS913T in  $\lambda$  gt11 were mixed with 0.5  $\mu$ g RNase A and 0.5  $\mu$ g DNase I and incubated at 37°C for one hour. The mixture was centrifuged at 5000xg for 10 min, the supernatant was freed by extraction with phenol and chloroform from protein, and the DNA was precipitated from the aqueous phase by addition of ethanol. The  $\lambda$ -DNA was dissolved in TE buffer (10 mM Tris pH = 7.5; 1 mM EDTA).

## b) PCR amplification of a TNF-BP sequence from a cDNA library.

16 individual reactions in which one of the 4 mixed oligonucleotides EBI-1639, EBI-1640, EBI-1641, EBI-1642

was respectively used as the first primer and one of the four mixed oligonucleotides EBI-1653, EBI-1654, EBI-1657, EBI-1658 as the second primer were carried out for the application of the PCR (Saiki et al., 1988) to DNA of the HS913T cDNA library. Each of these mixed oligonucleotides contains 48 different oligonucleotides of the same length. Amplification by means of PCR took place in 50  $\mu$ l reaction volume containing 250 ng  $\lambda$ -DNA of the cDNA library, 50 mM KCl, 10 mM Tris pH=8.3, 1.5 mM MgCl<sub>2</sub>, 0.01% gelatine, 0.2 mM of each of the 4 deoxynucleoside triphosphate(dATP, dGTP, dCTP, dTTP), each 200 pmol first and second primers, 1.25 units Taq polymerase [Perkin Elmer Cetus]. To avoid evaporation, the solution was overlaid with a few drops of mineral oil (0.1 ml). The PCR was carried out in a DNA Thermal Cycler (Perkin Elmer Cetus) as follows: The samples were heated to 94°C for 5 minutes to denature the DNA and subsequently subjected to 40 amplification cycles. One cycle consisted of a 40-second incubation at 94°C, a 2-minute incubation at 55°C and a 3-minute incubation at 72°C. At the end of the last cycle the samples were incubated at 72°C for another 7 minutes to ensure that the last primer extension is complete. After cooling to room temperature the samples were freed from protein with phenol and chloroform and the DNA was precipitated with ethanol.

5  $\mu$ l of each of the 16 PCR samples were applied to an agarose gel and the length of the amplified DNA fragments was determined after electrophoretic separation. The strongest DNA band, a fragment of 0.16 kb length, could be seen in the PCR samples which had been amplified with the oligonucleotide EBI-1653 as the first primer and one

of the oligonucleotides EBI-1639, EBI-1640, EBI-1641 or EBI-1642 as the second primer. Since the sample amplified with the primer pair EBI-1653 and EBI-1642 contained the largest amount of said 0.16 kb DNA fragment, this sample was selected for further processing.

Example 5:

Cloning and sequencing of a DNA fragment obtained by PCR amplification.

The resultant PCR product of the primers EBI-1642 and EBI-1653 was cut with BamHI and subsequently separated electrophoretically in an agarose gel (1.5% NuSieve GTG Agarose plus 1% Seakem GTG Agarose, FMC Corporation) according to size. The main band, a DNA fragment of 0.16 kb length, was electroeluted from the gel and precipitated with ethanol. This DNA fragment was ligated with BamHI/SmaI cut plasmid pUC18 (Pharmacia) and E. coli JM101 transformed with the ligation mixture. The plasmids prepared according to the minipreparation method were characterized by cutting with the restriction enzymes PvuII and EcoRI-BamHI and subsequent electrophoresis in agarose gels. The plasmid pUC18 contains two sites for PvuII which flank the polyconing site in a 0.32 kb DNA fragment. Very short DNA inserts at the polycloning site of the plasmid can be visualized more easily in the agarose gel after cutting with PvuII because the length increases by 0.32 kb. The DNA fragment ligated in the plasmid vector cut with BamHI and SmaI, including a few base pairs of the polylinker sequence, can be obtained by cutting with EcoRI and BamHI. A clone with the desired

insert was designated as pTNF-BP3B. After subcloning of an EcoRI-BamHI fragment the whole DNA insert of this clone was sequenced in M13mp18 (Pharmacia) after the modified dideoxy method with Sequenase (United States Biochemical Corporation).

An analysis of the DNA amplified by PCR showed the following sequence (only the non-coding strand is shown, the derived amino acid sequence thereabove):

|                                                            |    |    |    |
|------------------------------------------------------------|----|----|----|
|                                                            | 5  |    | 10 |
| Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys        |    |    |    |
| CAG GGG AAA TAT ATT CAC CCT CAA AAT AAT TCG ATT TGC        |    |    |    |
| 15                                                         |    | 20 | 25 |
| Cys Thr Lys Cys His Lys <u>Gly Thr Tyr Leu Tyr Asn Asp</u> |    |    |    |
| TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC        |    |    |    |
|                                                            | 30 |    | 35 |
| <u>Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg Glu Cys</u> |    |    |    |
| TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT        |    |    |    |

40

45

50

*Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn Asn Lys*

*GAG AGC' GGC TCC TTC ACA GCC TCA GAA AAC AAC AAG GAT CC*

The first 20 and the last 29 nucleotides (in italics) correspond to the sequences of the primer oligonucleotides EBI-1642 and the complement of EBI-1653. The amino acids 38 to 43 confirm the remaining sequence of the tryptic peptide 11.

Furthermore, the DNA fragment produced by means of PCR contains the sequence of the peptide of fraction 20 of the tryptic digest (amino acids 20 to 34, underlined).

Hence, this demonstrates that the clone pTNF-BP3B was derived from a cDNA which codes for TNF binding protein. pTNF-BP3B thus represents a probe, for instance for screening cDNA libraries for TNF-BP cDNAs.

#### Example 6:

##### Isolation of TNF-BP cDNA clones

About 720,000 phages of the HS913T cDNA library in  $\lambda$  gt11 were plated on E.coli Y1088 ( $\Delta$ lacU169, pro::TN5, tonA2, hsdR, supE, supF, metB, trpR, F-,  $\lambda$ -, (pMC9)) (about 60,000 phages per 14.5 cm Petri dish, LB agar: 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 1.5% agar, plating in top agarose: 10 g/l tryptone, 8 g/l NaCl, 0.8% agarose). Two nitrocellulose filter lifts were made from each plate. The filters were prewashed (16 hours at 65°C) in

50 mM        Tris/HCl    pH=8.0  
1 M NaCl  
1 mM EDTA  
0.1%        SDS

The filters were prehybridized at 65°C for two hours in:

6x    SSC (0.9 M NaCl, 0.09 M tri-Na-citrate) -  
5x    Denhardt's (0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA (= bovine serum albumin))  
0.1% SDS

Preparation of the radioactively labeled probe:

pTNF-BP 3B was cut twice with BamHI and EcoRI and the insert of about 0.16 kb was isolated. 0.6  $\mu$ g of the insert in 32  $\mu$ l was denatured at 100°C and primed with 60 pmol of EBI-1642 and EBI-1653 each by cooling to 80°C for 10 minutes and sudden cooling in iced water. After addition of

10  $\mu$ l  $\alpha$ -32p-dCTP (100  $\mu$ Ci, 3.7 MBq)  
 5  $\mu$ l 10x priming buffer (0.1 M Tris/HCl pH=8.0,  
 50 mM MgCL<sub>2</sub>)  
 2  $\mu$ l 1mM dATP, dGTP, dTTP each  
 1  $\mu$ l PolIK (Klenow fragment of the E.coli DNA  
 polymerase I, 5 units)

incubation was carried out at room temperature for 90 minutes. After heat inactivation (to 70°C for 10 minutes) the non-incorporated radioactivity was separated by chromatography via Biogel P6DG (Biorad) in TE buffer (10 mM Tris/HCl pH=8, 1 mM EDTA).  $65 \times 10^6$  cpm were incorporated.

The filters were hybridized in a total volume of 80 ml 6xSSC/5X Denhardt's/0.1% SDS plus heat-denatured hybridizing probe at 65°C for 16 hours.

The filters were washed in 6xSSC/0.01% SDS at room temperature for two times 30 minutes and in 2xSSC/0.01% SDS at room temperature once for 45 minutes and in 2xSSC/0.01% SDS at 65°C three times for 30 minutes. The filters were air-dried and subsequently exposed at -70°C on Amersham Hyperfilm for 16 hours using an intensifying screen. A total of 30 hybridizing plaques were identified ( $\lambda$ -TNF-BP #1-30).

The regions with the hybridizing plaques were cut out as precisely as possible, and the phages were eluted in 300  $\mu$ l SM buffer plus 30  $\mu$ l chloroform.

In the end 25 hybridizing phages were isolated ( $\lambda$ -TNF-BP #1-10, 12-24, 29, 30) by "plaque purification" (plating of about 200 phages per 9 cm Petri dish during the second pass and about 20 phages per 9 cm Petri dish during the third pass, filter lifts twice, preparation, hybridization and washing as described during the first screening).

Representation of the recombinant  $\lambda$ -DNA from the clones  $\lambda$ -TNF-BP #13, 15, 23, 30:

$2 \times 10^6$  phages were plated on E.coli Y1088 in top agarose (10 g/l tryptone, 8 g/l NaCl, 0.8% agarose) (14.5 cm Petri dish) with LB agarose (1.5% agarose, 0.2% glucose, 10 mM  $\text{MgSO}_4$ , 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) and incubated at 37°C for 6 hours. After cooling of the plates (at 4°C for 30 minutes) 10 ml  $\lambda$ -diluent (10 mM Tris/HCl pH=8.0, 10 mM  $\text{MgCl}_2$ , 0.1 mM ETA) were overlayed and elution was carried out at 4°C for 16 hours. The supernatant was transferred into 15 ml Corex tubes and centrifuged at 15000 rpm and 4°C for 10 minutes (Beckman J2-21 centrifuge, JA20 Rotor). The supernatant was decanted in 10 ml polycarbonate tubes and centrifuged at 50000 rpm, 20°C up to  $\omega^2 t = 3 \times 10^{10}$  (Beckmann L8-70, 50 Ti Rotor). The pellet was resuspended in 0.5 ml  $\lambda$ -diluent and transferred in Eppendorf tubes (1.4 ml). After addition of 5  $\mu\text{g}$  RNase A and 0.5  $\mu\text{g}$  DNaseI and incubation at 37°C for 30 minutes and addition of 25  $\mu\text{l}$  0.5 M EDTA, 12.5  $\mu\text{l}$  1 M Tris/HCl pH=8.0, 6.5  $\mu\text{l}$  20% SDS there was another incubation at 70°C for 30 minutes. The  $\lambda$ -DNA was purified by phenol/chloroform extraction and precipitated with ethanol. Finally the DNA was dissolved in 100  $\mu\text{l}$  TE buffer.

## Example 7:

## Subcloning and sequencing of TNF-BP cDNA clones 15 and 23

To specify the cDNAs of the clones  $\lambda$ TNF-BP15 and  $\lambda$ TNF-BP23, which had shown the strongest signals during hybridization, the cDNA inserts were cut out with EcoRI from the  $\lambda$ -DNA, eluted from an agarose gel after electrophoretic separation and precipitated with ethanol. The DNA fragments of 1.3 kb (of  $\lambda$ TNF-BP15) and 1.1 kb (of  $\lambda$ TNF-BP23) were cut with EcoRI and ligated with alkaline phosphatase of calf intestine-dephosphorylated plasmid vector pT7/T3 $\alpha$ -18 (Bethesda Research Laboratories) with T4 DNA ligase and E.coli JM101-transformed. From individual bacterial colonies which showed no blueness after selection on agarose plates with ampicillin and X-gal, plasmid DNA was made in a miniprep method, and the presence and orientation of the cDNA insert were determined by cutting with EcoRI and HindIII. Plasmids which contained the EcoRI insert of the phages  $\lambda$ TNF-BP15 and  $\lambda$ TNF-BP23 oriented in such a manner that the end corresponding to the 5' end of the mRNA faced the T7 promoter were named pTNF-BP15 and pTNF-BP23, respectively. The EcoRI inserts of  $\lambda$ TNF-BP15 and  $\lambda$ TNF-BP23 were also ligated in M13mp19 vector, which had been cut with EcoRI and dephosphorylated, and E.coli JM101 transformed. Single-strand DNA was prepared from some randomly selected M13 clones and used as a template for the sequencing according to the dideoxy method.

On M13 clones which contained the cDNA inserts in the opposite orientation, both DNA strands were completely sequenced with the universal sequencing primer and with specifically

synthetized oligonucleotide primers binding to the cDNA insert.

The complete nucleotide sequence of 1334 bases of the cDNA insert of  $\lambda$ TNF-BP15 and pTNF-BP15 is illustrated in Fig. 1. The bases 1-6 and 1328-1334 correspond to the EcoRI linkers which had been added to the cDNA during the construction of the cDNA library. The nucleotide sequence of the cDNA insert of  $\lambda$ TNF-BP23 corresponds to that of  $\lambda$ TNF-BP15 (bases 22-1100), flanked by EcoRI liners.

The clone  $\lambda$ TNF-BP30 was also examined; its sequence corresponds to  $\lambda$ TNF-BP15 except for the difference that the sequence has a deletion of 74 bp (nucleotide 764 to 837).

#### Example 8

##### Construction of the expression plasmids pAD-CMV1 and pAD-CMV2

A new plasmid which has a multicloning site for the directed insertion of heterologous DNA sequences and can be propagated in *E.coli* by means of ampicillin resistance with a high copy number was constructed from parts of the expression plasmids pCDM8 (Seed and Aruffo, 1987. Seed, 1987; Invitrogen), pW2gptDHFR20 (EP-A1 0321 842) and the plasmid Bluescript SK+ (Short et al., 1988; Stratagene). The intergenic region of M13 permits the production of single-stranded plasmid DNA by means of superinfection of the transformed bacteria with a helper phage (e.g. R408 or M13K07) for facilitated sequencing and mutagenesis of the plasmid DNA. The T7 promoter which precedes the multicloning site permits the production of RNA transcripts in vitro. The expression of heterologous genes,

directed by the cytomegalovirus (CMV) promoter/enhancer (Boshart et al., 1985), takes place in mammalian cells. The SV40 replication origin permits the autonomous replication of the expression plasmid to high copy numbers in suitable cell lines (e.g. SV40-transformed cells such as COS-7, adenovirus-transformed cell line 293 (ATCC CRL1573) and thus high rates in transient expression. For the production of permanently transformed cell lines and the subsequent amplification of the expression cassette by means of methotrexate, a modified hamster minigene (promoter with coding region and the first intron) for dihydrofolate reductase (DHFR) serves as selection marker.

a) Preparation of the vector and promoter portions by PCR

The plasmid Bluescript SK+ was linearized with HindIII and 5 ng DNA was inserted into a 100  $\mu$ l PCR batch (reaction buffer: 50 mM KCl, 10 mM Tris-Cl pH=8.3, 1.5 mM MgCl<sub>2</sub>, 0.01% (w/v) gelatine, 0.2 mM of the four deoxynucleotidetriphosphates (dATP, dGTP, dCTP, dTTP), 2.5 units Taq polymerase per 100  $\mu$ l). 50 pmol of the synthetic oligonucleotides EBI-1786 (5'-GGAATTCAGCCTGAATGGCGAATGGG-3'; binds just outside of M13 ori-region in Bluescript pos. 475, independent of M13 ori-orientation) and EBI-1729 (5'-CCTCGAGCGTTGCTGGCGTTTTTCC-3'; binds to Bluescript at pos. 1195 before ori, corresponds to the beginning of the Bluescript sequence in pCDM8, 6 bases 5' give XhoI) were respectively used as primers. After 5 minutes of denaturation at 94°C the PCR was performed over 20 cycles (40 sec at 94°C, 45 sec at 55°C, 5 min at 72°C, Perkin Elmer Cetus Thermal Cycler). The oligonucleotides flank the intergenic region of M13 and the replication origin (ori) with the interposed gene for the  $\beta$ -lactamase. At the same time, an

XhoI is produced at the end of the replication origin and an EcoRI site at the other end. The reaction mixture was freed from protein by extraction with phenol/chloroform and the DNA was precipitated with ethanol. The resultant DNA was cut with XhoI and EcoRI and a fragment with 2.3 kb was isolated after electrophoresis in an agarose gel.

5 ng with SacII linearized plasmid pCDM8 were amplified by PCR with the oligonucleotides EBI-1733 (5'-GGTCGACATTGA-TTATTGACTAG-3'; binds to CMV promoter region (pos. 1542) of pCDM8, corresponds to pos. 1 in pAD-CMV, SalI site for cloning) and EBI-1734 (5'-GGAATTCCCTAGGAATACAGCGG-3'; binds to polyoma origin of 3'SV40 poly(A) region in pCDM8 (pos. 3590)) under identical conditions as described for Bluescript SK+. The oligonucleotides bind at the beginning of the CMV-promoter/enhancer sequence and produce an SalI site (EBI-1733) or bind at the end of the SV40 polyadenylation site and produce an EcoRI site (EBI-1734). The PCR product was cut with SalI and EcoRI and a DNA fragment of 1.8 kb was isolated from an agarose gel.

The two PCR products were ligated with T4 DNA ligase, transformed with the resultant ligation product E.coli HB101 and plasmid DNA amplified and prepared according to standard methods. The plasmid of the desired nature (see Fig. 3) was named pCMV-M13.

The SV40 replication origin (SV40 ori) was isolated from the plasmid pSV2gptDHFR20 (EP-A1 0321842). To this end the plasmid was cut with HindIII and PvuII twice and the DNA ends were made blunt by the subsequent treatment of the large fragment of the E.coli DNA polymerase (Klenow enzyme) in the presence

of the four deoxynucleotidetriphosphates. A resultant 0.36 kb DNA fragment was isolated from an agarose gel and ligated in EcoRI-linearized pCMV-M13. A plasmid obtained after transformation of *E. coli* HB101 with the SV40 ori in the same orientation as the  $\beta$ -lactamase gene and the CMV promoter was named pCMV-SV40. The construction of this plasmid is shown in Fig. 3.

#### b) Mutagenesis of the DHFR gene

To prepare an expression plasmid with a multiple multicloning site, two restriction sites were removed from the DHFR minigene by directed mutagenesis and three restriction sites by deletion. To this end a 1.7 kb BglII fragment, which contains the whole coding region of the hamster DHFR gene, was cloned from the plasmid pSV2gptDHFR20 into the BglII site of the plasmid pUC219 (IBI), and the plasmid pUCDHFR was obtained. *E. coli* JM109 (Stratagene) cells transformed with pUCDHFR were infected with about the 40-fold surplus of the helper phage R408 (Stratagene) and shaken in LB medium at 37°C for 16 hours. Single-stranded plasmid DNA was isolated from the bacterial supernatant.

The directed mutagenesis was performed in two successive steps, the in vitro mutagenesis system RPN1523 (Amersham) being used. The EcoRI site located at the beginning of exon 2 was destroyed by exchange of a base of GAATTC to GAGTTC. This base exchange does not lead to any change in the coded amino acid sequence and corresponds to the nucleotide sequence in the natural murine DHFR gene (McGrogan et al., 1985, Mitchell et al., 1986). An oligonucleotide (antisense orientation) of the sequence 5'-GTACTTGAAC TCGTTCCTG-3' (EBI-1751) was used for

mutagenesis. A plasmid with the desired mutation was prepared as a single-strand DNA, as described above, and the PstI site positioned in the first intron was removed by mutagenesis with the oligonucleotide EBI-1857 (antisense orientation 5'-GGCAAGGGCAGCAGCCG-3') from CTGCAG into CTGCTG. The mutations were confirmed by sequencing and the resultant plasmid was named pUCDHFR-Mut2.

The 1.7 kb BglII fragment was isolated from the plasmid pUCDHFR-Mut2 and ligated in plasmid pSV2gptDHFR20 cut twice with BglII and BamHI. After transformation of E.coli, ( ) plification and DNA isolation, a plasmid of the desired constitution was obtained and named pSV2gptDHFR-Mut2. A 0.12 kb DNA fragment following the BIII site was removed by cutting with BamHI in the 3'-noncoding region of the DHFR gene, the DNA fragment additionally containing a KpnI site. The recognition sequences for these two enzymes were also destroyed by linking the overhanging DNA ends obtained with BglII and BamHI.

The plasmid pCMV-SV40 was cut twice with EcoRI and BamHI; the DNA ends were subsequently made blunt with the Klenow enzyme. The DNA was purified by extraction with phenol/chloroform and ethanol precipitation, subsequently dephosphorylated by ( ) cubation with alkaline phosphatase and the 4.4 kb long vector DNA was isolated from an agarose gel.

The plasmid pSV2gptDHFR-Mut2 (Fig. 4) was cut twice with EcoRI and PstI and the DNA ends were made blunt by incubation at 11°C for 20 minutes with 5 units T4 DNA polymerase (50 mM Tris- HCl pH=8.0, 5 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 0.1 mM of each of the four deoxynucleotide triphosphates, 50 µg/ml

bovine serum albumin). The 2.4 kb long DNA fragment with the mutated DHFR gene was isolated from an agarose gel and ligated with the pCMV-SV40 prepared as described above. A plasmid obtained after transformation of E.coli, which contained the DHFR gene in the same orientation as the CMV promoter, was named pCMV-SV40DHFR.

In the last step the 0.4 kb "Stuffer" fragment after the CMV promoter that still derived from the initial plasmid pCDM8 was exchanged for a multicloning site. To this end the plasmid pCMV-SV40DHFR was cut twice with HindIII and XbaI and the vector portion was isolated from an agarose gel. The multicloning site, formed from the two oligonucleotides EBI-1823 (5'-AGCTTCTGCAGGTCGA-CATCGATGGATCCGGTACCTCGAGCGGCCGCGAATTCT-3') and EBI-1829 (5'-CTAGAGAATTCGCGGCCGCTCGAGGTACCGGATCCATCGATGTCGACCTGCAGA-3') contains restriction sites for the enzymes PstI, SalI, ClaI, BamHI, KpnI, XhoI, NotI and EcoRI, inclusive of the ends compatible for the cloning in HindIII - XbaI.

1  $\mu$ g each of the two oligonucleotides was incubated in 20  $\mu$ l reaction buffer (70 mM Tris-Cl pH=7.6, 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 0.1 mM ATP) with 5 units T4 polynucleotide kinase at 37°C for one hour to phosphorylate the 5' end. The reaction was stopped by heating to 70°C for 10 minutes and the complementary oligonucleotides were hybridized with one another by incubating the sample at 56°C for another 10 minutes and subsequently cooled slowly to room temperature. 4  $\mu$ l of the hybridized oligonucleotides (100 ng) were ligated with about 100 ng plasmid vector and E.coli HB101 transformed. A plasmid which could be linearized with the enzymes of the multicloning site (except for NotI) was named pAD-CMV1. Out of

the many tested clones, none could be identified whose plasmid could be cut with NotI. The sequencing always showed the deletion of a few bases within the NotI recognition sequence.

The expression plasmid pAD-CMV2 which contains the restriction sites within the multicloning site in reverse order was produced in the same manner with the oligonucleotide pair EBI-1820 (5'-AGCTCTAGAGAATTCGCGGCCGCTCGAGGT-ACCGGATCCATCGATGTCGACCTGCAGAAGCTTG-3') and EBI-1821 (5'-CTAGCAAGCTTCTGCAGGTGACATCGATGGATCCGGTACCTCGAGCG-GCCGCGAATTCTCTAG-3'). This led to the plasmid pAD-CMV2 which could be linearized with all restriction enzymes, including NotI.

The nucleotide sequence of the 6414 bp large plasmid pAD-CMV1 (Fig. 5) is completely shown in Fig. 6.

The segments on the plasmid (indicated in the numbering of the bases) correspond to the following sequences:

|                |                                                                                   |
|----------------|-----------------------------------------------------------------------------------|
| 1-21           | EBI-1733, beginning CMV enhancer - promoter<br>(from CDM8)                        |
| 632-649        | T7 promoter                                                                       |
| 658-713<br>( ) | multicloning site (HindIII to XbaI from<br>EBI-1823, EBI-1829)                    |
| 714-1412       | SV40 intron and polyadenylation site<br>(from CDM8)                               |
| 1413-2310      | 5'-noncoding region and promoter of the<br>hamster DHFR gene (from pSV2gptDHFR20) |
| 2311-2396      | hamster DHFR: exon 1                                                              |
| 2516           | A to T mutation destroys PstI site in DHFR<br>intron 1                            |

|           |                                                                 |
|-----------|-----------------------------------------------------------------|
| 2701-3178 | DHFR exons 2-6 (coding region)                                  |
| 2707      | A to G mutation destroys EcoRI site                             |
| 3272-3272 | deletion between BglII and BamHI in DHFR<br>3'-noncoding region |
| 3831      | end DHFR gene (from pSV2gptDHFF20)                              |
| 3832-4169 | SV40 ori (from pSV2gptDHFR20)                                   |
| 4170-4648 | M13 ori (from pBluescript SK+)                                  |
| 4780-5640 | $\beta$ -lactamase (coding region)                              |
| 6395-6415 | EBI-1729, end of the pBluescript vector<br>sequence.            |

The production of the plasmids pAD-CMV1 and pAD-CMV2 is shown in Fig. 5.

#### Example 9

Construction of the plasmid pADTNF-BP for the expression of the soluble form of TNF-BP.

To produce the secreted form of TNF-BP directly, a translation stop codon was introduced into the cDNA coding for part of the TNF receptor (cf. Example 7; designated as TNF-R cDNA in the following) after the codon of the C-terminal amino acid of the natural TNF-BP (AAT, Asn-172; corresponds to pos. 201 in Fig. ( )). The protein synthesis is thereby interrupted at this place and permits the direct secretion of TNF-BP into the cell supernatant without the need for passing a subsequent, possibly speed-determining reaction of a proteolytic cleavage in segments of the TNF receptor situated in C-terminal direction.

At the same time, the 5'-noncoding region of the TNF-R cDNA was shortened with the introduction of the stop codon by means of PCR to remove the translation start codon of another open reading frame (bases 72-203 in Fig. 9) positioned 5' from that of the TNF-R, and a BamHI and EcoRI site was introduced at the 5'-terminus and 3'-terminus, respectively, of the cDNA.

100 ng of XmnI-linearized plasmid pTNF-BP15 (cf. Example 7) were amplified with 50 pmol each of the oligonucleotides EBI-1986 (sense, 5'CAGGATCCGAGTCTCAACCCTCAAC-3') and EBI-1929 (antisense, 5'-GGGAATTCCTTATCAATTCTCAATCTGGGGTAGGCACAACCTTC-3'); incorporation of two stop codons and of an EcoRI site) in a 100  $\mu$ l PCR batch over 10 cycles. The cycle conditions were 40 seconds at 94°C, 45 seconds at 55°C and 5 minutes at 72°C. After the last cycle incubation was continued at 72°C for another 7 minutes and the reaction was stopped by extraction with phenol/chloroform. The DNA was precipitated with ethanol and subsequently cut twice with BamHI and EcoRI. The resultant 0.75 kb DNA fragment was isolated from an agarose gel and cloned in plasmid pT7/T2 $\alpha$ -19 (BRL) cut twice with BamHI and EcoRI. One of the resultant plasmids with respect to which it had been found by virtue of the sequencing of the whole insert that it had the desired sequence was named pTNF-BP.

pTNF-BP was cut with BamHI and EcoRI and the 0.75 kb DNA insert was cloned into the expression plasmid pAD-CMV1 cut with BamHI and EcoRI. A resultant plasmid of the desired composition was named pADTNF-BP (Fig. 7A).

#### Example 10

Construction of the plasmid pADBTNF-BP for the expression of the soluble form of TNF-BP.

For another variant of an expression plasmid for the production of secreted TNF-BP, the 5'-noncoding region of the TNF-R cDNA was replaced by the 5'-noncoding region of the human  $\beta$ -globin mRNA. The reason therefor was the finding that the nucleotide sequence directly before the translation start codon of the TNF-R sequence greatly differs from the consensus sequence (Kozak, 1987) found for the efficient expression of eukaryotic genes, whereas the 5'-noncoding region of the  $\beta$ -globin mRNA corresponds to this consensus sequence very well (Lawn et al., 1980). The TNF-R sequence was modified in a PCR by means of the oligonucleotide EBI-2452 (5'-CACAGTCGACTTACATTTGCTTCTGACACAACCTGTGTTCCTAGCAACCTCAAACAGACACCATGGGCCTCTCCACCGTGC-3'), which after a SalI restriction site contained the authentic 5'-noncoding sequence according to the human  $\beta$ -globin mRNA sequence, followed by 20 bases of the coding region of TNF-BP. 100 ng of EcoRI-linearized plasmid pTNF-BP were amplified in 100  $\mu$ l reaction batch with 50 pmol each of the oligonucleotides EBI-2452 and EBI-1922 (antisense, 5'-GAGGCTGCAATTGAAGC-3'; binds to the huTNF-R sequence at pos. 656) in 20 PCR cycles (40 sec at 94°C, 45 sec at 55°C, 90 sec at 72°C). After purification of the PCR product by extraction with phenol/chloroform and ethanol precipitation the DNA was cut twice with SalI and BglIII and the resultant 0.51 kb DNA fragment was isolated from an agarose gel. The corresponding part of the TNF-R sequence was removed from the plasmid pTNF-BP by cutting with SalI and BglIII, the 3.1 kb long plasmid part was isolated from an agarose gel and ligated with the 0.51 kb long PCR product. After transformation of E.coli seven of the plasmids obtained

were sequenced. One of these plasmids exactly contained the desired sequence. This plasmid was named pBTNF-BP.

The whole SalI - EcoRI insert of pBTNF-BP was cloned in the also cut expression plasmid pAD-CMV1 and the resultant plasmid was named pADBTNF-BP (Fig. 7B).

#### Example 11

##### Isolation of rat TNF-R cDNA clones

(First of all a rat brain cDNA was produced by analogy with the HS913T cDNA library (cf. Example 4) from the rat glia tumor cell line C6 (ATCC no. CCL107) in  $\lambda$ -gt11.

600,000 phages of the rat brain cDNA library in  $\lambda$ -gt11 were screened by hybridization as described in Example 6. The purified EcoRI insert of pTNF-BP30 (cf. Example 6) was used as the probe. About 100 ng DNA were radioactively labeled with 1  $\mu$ g Random Hexamer Primer instead of the specific oligonucleotides, as described in Example 6, with [ $\alpha$ -<sup>32</sup>P]dCTP. 25-10<sup>6</sup> were incorporated. The filters were hybridized under the same conditions as in Example 6. The filters were washed twice at room temperature in 2xSSC/0.1% SDS for 30 minutes and three times at 65°C in 2xSSC/0.1%SDS for 30 minutes and twice at 65°C in 0.5xSSC/0.5%SDS for 30 minutes. The air-dried filters were subsequently exposed on Kodak XAR X-ray film for 16 hours using an intensifying screen at -70°C. A total of 10 hybridizing plaques were identified and isolated by plaque purification. Following plaque purification conducted three times, three  $\lambda$ -clones ( $\lambda$ -ratTNF-R # 3, 4, 8) were isolated in the end and the phage DNA represented, as described.

After cutting of the  $\lambda$ -DNA with EcoRI and separation in an agarose gel the length of the cDNA inserts was determined to be 2.2 kb for the clones raTNF-R3 and raTNF-R8 and 2.1 kb for clone raTNF-R4. The EcoRI inserts of the clones  $\lambda$ raTNF-3 and 8 were cloned in M13mp19 cut in the same way and the DNA sequence was determined with universal sequencing primers and specifically synthesized oligonucleotide primers.

The complete nucleotide sequence of raTNF-R8 is illustrated in Fig. 8. The first bases and the seven last bases correspond to the EcoRI linkers which were added during production of the cDNA library.

#### Example 12

Isolation of a clone containing the complete cDNA coding for the human TNF receptor.

The complete cDNA of the rat TNF-R facilitated the search for the still missing 3' part of the human TNF-R cDNA.

As the probe for the hybridizing process, the 0.4 kb long PCR product of the primer EBI-2316 (5'-ATTCGTGCGGCGCCTAG-3'; binds to TNF-R with the second base of EcoRI, stops at the TNF-R cDNA) and EBI-2467 (5'-GTCGGTAGCACCAAGGA-3'; binds about 400 bases before poly(A) to cDNA clone, corresponds to pos. 1775 in raTNF-R) was used with  $\lambda$ raTNF-R8 as the template. This DNA fragment corresponds to the region of the rat TNF-R cDNA of which it was assumed that it corresponds to the one which follows the internal EcoRI site in human TNF-R.

$2.5 \times 10^6$  cpm of the ratTNF-R probe were used to hybridize 600,000 plaques of the HS913 cDNA library. The hybridization conditions corresponded to the ones indicated in Example 6. The filters were washed twice at room temperature in  $2 \times \text{SSC}/0.1\% \text{SDS}$  for 30 minutes and twice at  $65^\circ\text{C}$  in  $2 \times \text{SSC}/0.1\% \text{SDS}$  for 30 minutes, dried in air and exposed on Kodak XAR X-ray film using an intensifying screen at  $-70^\circ\text{C}$  for 3 days. Six positive plaques were identified, plaques were purified in two further rounds and  $\lambda$ DNA was prepared ( $\lambda$ -TNF-R #2, 5, 6, 8, 11, 12). After cutting of the  $\lambda$ DNA with Eco RI all of the clones had a DNA band with a length of about 0.8 kb.  $\lambda$ -TNF-R2 and 11 additionally contained an EcoRI fragment with 1.3 kb. The two EcoRI inserts of  $\lambda$ -TNF-R2 were subcloned in the EcoRI site of plasmid pUC218 (IBI) and subsequently sequenced. The sequence of the 1.3 kb EcoRI fragment corresponded to the one of cDNA clone pTNF-BP15, the 0.8 kb EcoRI fragment corresponded to the 3' segment of the TNF-R mRNA and contained a poly(A) tail with 16 A residues before the EcoRI linker sequence. Hence,  $\lambda$ -TNF-R2 contained the complete coding region of the human TNF-R, represented in Fig. 9.

### Example 13

Construction of the plasmids pADTNF-R and pADBTNF-R for the expression of the whole human TNF receptor

First a plasmid in which the 5'-noncoding region of pTNF-BP15 was shortened, but in which, in contrast to the plasmids described in example 9, the 3' end of pTNF-BP15 was maintained, was first constructed in a way similar to the one described in Example 9 for pTNF-BP and pADTNF-BP, respectively. Under identical conditions as in Example 9 pTNF-

BP15 was amplified by PCR with the oligonucleotide EBI-1986 and the M13 -40 universal primer (5'-GTTTTCCTCCAGTCACGAC-3'). The PCR product was cut twice with BamHI and EcoRI and cloned into plasmid pT7/T3 $\alpha$ -19. One of the plasmids obtained was called pTNF-BP15B.

pTNF-BP15B was cut with BamHI and EcoRI and the 1.26 kb DNA insert was cloned in expression plasmid pAD-CMV1 cut with BamHI and EcoRI. A resultant plasmid of the desired composition was named pADTNF-BP15.

This plasmid was linearized with EcoRI and the 0.8 kb EcoRI fragment, isolated from  $\lambda$ TNF-R2, was cloned into the site. After transformation of E.coli some randomly isolated plasmids were checked by cutting with different restriction enzymes as to the correct orientation of the EcoRI fragment used. A plasmid designated as pADTNF-R (Fig. 7C) was even checked more closely as to its correct orientation by sequencing the insert starting from the 3' end of the inserted cDNA with the oligonucleotide EBI-2112 (5'-GTCCAATTATGTCACACC-3') which binds to the plasmid pAD-CMV1 and its derivatives after the multicloning site.

Another expression plasmid in which the 5'-noncoding region of the TNF-R is replaced by the one of  $\beta$ -globin was constructed. Plasmid pADBTNF-BP was completely cut with BglII to remove the 1.1 kb BglII fragment, the DNA ends were subsequently dephosphorylated with alkaline phosphatase from calf intestine and the plasmid vector (5.9 kb) was isolated with the  $\beta$ -globin 5'-noncoding region of the  $\beta$ -globin gene and the 5' part of the TNF-R coding region from an agarose gel. Plasmid pADTNF-R was cut with BglII and the 2.5 kb DNA fragment containing the

3' segment of the TNF-R cDNA up to the promoter region of the following DHFR gene was isolated from an agarose gel and cloned into the previously prepared plasmid vector. A plasmid obtained after transformation of E.coli with the BglII fragment inserted in correct orientation was named pADBTNF-R (Fig. 7D).

#### Example 14

##### Expression of soluble TNF-BP in eukaryotic cell lines

##### ELISA

The detection of TNF-BP by means of ELISA was carried out in this example as follows:

96-well microtiter plates were coated per well with 50  $\mu$ l 1:3000 diluted polyclonal rabbit serum (polyclonal rabbit antibody prepared by precipitation of antiserum with ammonium sulfate, final concentration 50% saturation) against natural TNF-BP at 4°C for 18 hours, once washed with 0.05% Tween-20 in PBS, and free binding sites were blocked with 150-200  $\mu$ l 0.5% bovine serum albumin, 0.05% Tween-20 in PBS (PBS/BSS/Tween) at room temperature for one hour. The wells were once washed with 0.05% Tween-20 in PBS and 50  $\mu$ l cell supernatant or known amounts of natural TNF-BP (cf. Tables 3 and 4) and 50  $\mu$ l of a 1:10,000 dilution of a polyclonal mouse serum were mixed with TNF-BP and incubated at room temperature for two hours. The wells were subsequently washed three times with 0.05% Tween-20 in PBS, and 50  $\mu$ l rabbit anti-mouse Ig-peroxidase conjugate (Dako P161; 1:5000 in PBS/BSA/Tween) were added and incubated at room temperature for another two hours. The wells were

washed with Tween/PBS three times and the staining reaction was performed with orthophenylene diamine (3 mg/ml) and Na perborate (1 mg/ml) in 0.067 M potassium citrate pH 5.0, 100  $\mu$ l/well, at room temperature for 20 minutes under protection from light. After addition of 100  $\mu$ l 4N H<sub>2</sub>SO<sub>4</sub> the color intensity was measured at a wavelength of 492 nm in a microfilm plate photometer.

b) Transient expression of soluble TNF-BP in eukaryotic cell lines

About  $10^6$  cells (COS-7) per 80 mm Petri dish were incubated with 10% heat-inactivated fetal calf serum 24 hours before transfection in RPMI-1640 medium and incubated at 37°C in 5% CO<sub>2</sub> atmosphere. The cells were removed with a rubber scraper from the Petri dish and centrifuged off at 1200 rpm at room temperature for 5 minutes (Heraeus Minifuge, Ausschwing-Rotor 3360), washed once with 5 ml of serum-free medium, centrifuged at 1200 rpm for 5 minutes and suspended in 1 ml medium, mixed with 250  $\mu$ g/ml DEAE-dextrane and 10  $\mu$ g plasmid DNA (cf. Table 3, purified by CsCl density gradient centrifugation performed twice). The cells were incubated at 37°C for 40 minutes, washed once with 5 ml medium with 10% calf serum and suspended in 5 ml medium with 100  $\mu$ g/ml chloroquine. The cells were incubated at 37°C for one hour, washed once with medium and incubated at 37°C with 10 ml fresh medium. After 72 hours the cell supernatant was harvested and used for detection of the secreted TNF-BP.

Table 3:

| Cell Line       | COS-7     |
|-----------------|-----------|
| -----           |           |
| without plasmid | < 5 ng/ml |
| pADTNF-BP       | 7.5 ng/ml |
| pADBTNF-BP      | 146 ng/ml |

c) Production of permanently TNF-BP produced cell lines

The dihydrofolate reductase(DHFR)-deficient hamster ovarial (cl) line CHO DUKX BII (Urlaub and Chasin, 1980) was transfected with plasmid pADBTNF-BP by means of calcium phosphate precipitation (Current Protocols in Molecular Biology, 1987). Four densely grown cell culture vials (25 cm<sup>2</sup>, 5 ml culture medium per vial) were transfected with 5 µg DNA each; after incubation at 37°C for four hours the medium was removed and replaced by 5 ml selection medium each (MEM alpha medium with 10% dialyzed fetal bovine serum). After overnight incubation the cells were removed by means of trypsin solution; the cells from the vials were distributed over two 96-well tissue culture plates (100 µl/well in selection medium). Fresh medium was added in approximately weekly intervals. After about four weeks cell clones could be observed in 79 wells. The supernatants were assayed in the ELISA for TNF-BP activity. 37 supernatants showed activity in the ELISA. The results of the ELISA of some positive clones are shown in Table 4.

Table 4:

| Sample                                  | Absorption at 492 nm |
|-----------------------------------------|----------------------|
| -----                                   |                      |
| TNF-BP Standard                         |                      |
| 1 ng/ml                                 | 0.390                |
| 10 ng/ml                                | 1.233                |
| 100 ng/ml                               | 1.875                |
| -----                                   |                      |
| Culture medium (negative control) 0.085 |                      |
| -----                                   |                      |
| Clone                                   |                      |
| A1G3                                    | 0.468                |
| A2F5                                    | 0.931                |
| A3A212                                  | 0.924                |
| A4B8                                    | 0.356                |
| A5A12                                   | 0.806                |
| A5B10                                   | 0.915                |
| A5C1                                    | 0.966                |
| -----                                   |                      |

Example 15:

RNA Analysis (Northern blot) of the human TNF receptor

1  $\mu$ g poly(A)<sup>+</sup> RNA (isolated from HS913T (fibrosarcoma), placenta and spleen were respectively electrophoretically separated in a 1.5% vertical agarose gel (10mM Na phosphate buffer pH=7.0, 6.7% formaldehyde). A kilobase ladder (Bethesda

Research Laboratories) radioactively labeled by filling reaction with ( $\alpha$ - $^{32}$ P)dCTP and Klenow enzyme was used as size marker. The formaldehyde was removed from the gel by soaking in water and the RNA was transferred in 20x SSC to a nylon membrane (Genescreen plus, NEN-DuPont). The RNA was covalently bound by UV irradiation (100 seconds) to the membrane. The membrane was prehybridized at 65°C in Church buffer (Church and Gilbert, 1984) (0.5 M Na phosphate pH=7.2, 7% SDS, 1 mM EDTA) at 65°C for 2 hours and hybridized in fresh Church buffer with  $3 \times 10^6$  cpm P-32 labeled DNA probe (EcoRI insert of pTNF-BP30) at 65°C for 19 hours. The filter was washed at room temperature in wash buffer (40 mM Na phosphate pH=7.2, 1% SDS) three times for 10 minutes and then washed at 65°C in wash buffer four times for 30 minutes and exposed on Kodak XAR X-ray film for 18 hours using an intensifying screen at -70°C. The autoradiogram (Fig. 10) shows a singular RNA band of a length of 2.3 kb for the human TNF receptor in the analyzed tissues and the cell line HS913T.

#### Example 16:

##### Expression of the TNF receptor

For the transient expression  $5 - 10 \times 10^7$  COS-7 cells were incubated for 40 minutes with 10  $\mu$ g pADTNF-R-plasmid DNA in a solution containing 250  $\mu$ g/ml DEAE dextrane and 50  $\mu$ g/ml chloroquine. pADCMV-1-DNA was used as the control. After transfection the cells were washed and subsequently cultivated for 48 h. The expression of the TNF receptor was detected by binding of  $^{125}$ I-TNF. For the binding test the cells were washed, incubated for 1 h at 4°C with 10 ng  $^{125}$ I-TNF (specific radioactivity 38,000 cpm/ng) with or without a 200-fold

surplus of unlabeled TNF, washed and the radioactivity bound to the cells was measured in a gamma counter. The specific binding was 2062 cpm in the control sample, 6150 cpm in the samples transformed with TNF receptor DNA (the values are expressed as mean bound cpm; the standard deviation, determined in parallel tests, is taken into account. Unspecific underground in the presence of unmarked TNF was deducted from the values).

## Literature:

- Aggarwal, B.B., et al., 1985, Nature 318, 655-667
- Beutler B., et al., 1988, Ann. Rev. Biochem. 57, 505-18
- Beutler, B., et al., 1985, Nature 316, 552 - 554
- Boshart, M., et al., 1985, Cell 41, 521-530
- Carswell, E.A., et al., 1975, Proc.Natl.Acad.Sci.25,  
3666-3670
- Cerami, A., et al., 1988, Immunol. Today 9, 28-31
- Church and Gilbert, 1984, Proc.Natl.Acad.Sci. 81,  
1991-1995
- Creasey, A.A., et al., 1987, Proc.Natl.Acad.Sci. 84,  
3293-3297
- Engelmann, H., et al., 1990, J.Biol.Chem.265, 1531-1536
- Frohman, M.A., et al., 1988, Proc.Natl.Acad.Sci. 85,  
8998-9002
- Gullberg, U., et al., 1987, Eur. J. Haematol. 39,  
241-251
- Hsieng, S.L., et al., 1988, J. Chromatography 447,  
351-364
- Klebanoff, S.J., et al., 1986, J. Immunol. 136,  
4220-4225
- Kozak M., 1987, Nucleic Acids Res. 15, 8125-8148
- Laemmli, U.K., 1970, Nature 227, 680-4
- Lawn, et al., 1980, Cell 21, 647-651
- Locksley, R.M., et al. 1987. J.Imunol. 139, 1891-1895
- Mahoney, J.R., et al., 1985, J. Immunol. 134,  
1673-1675
- Maniatis, T., et al., 1982, Molecular Cloning A  
laboratory Manual. Cold Spring Harbor  
Laboratory, 474
- McGrogan, M., et al., 1985, J.Biol.Chem. 260, 2307-2314
- Mestan, J., et al., 1986, Nature 323, 816-819
- Mitchell, P.J., et al., 1986, Mol.Cell.Biol. 6, 425-440

- Oakley, B.R., et al., 1986, *Analyt. Biochem.* 105, 361-363
- Old, L.J., 1987, *Nature* 326, 330-331
- Oliff A., et al., 1987, *Cell* 555-63
- Olsson I., et al., 1988, *Eur.J. Haematol.* 41, 414 - 420
- Olsson I., et al., 1989, *Eur.J. Haematol.* 42, 270 - 275
- Pieler Ch., 1987, *Dissertation, Universität Wien*
- Piguet, P.F., et al., 1987, *Immunobiol.* 175, 27
- Saiki, R.K., 1988, *Science* 239, 487-491
- Sanger et al., 1977, *Proc.Natl.Acad.Sci.* 74, 5463-5467
- Seckinger P., et al., 1988, *J. Exp. Med.*, 1511-16
- Seckinger, P., et al., 1987, *J. Immunol.* 139, 1546-1549
- Seed und Aruffo, 1987, *Proc.Natl.Acad.Sci.* 84, 8573-8577
- Seed, B., 1987, *Nature* 329, 840-842
- Shalaby, M.R., et al., 1985, *J. Immunol.* 135, 2069-2073
- Short, J.M., et al., 1988, *Nucl.Acids Res.* 11, 5521-5540
- Staden, R., 1982, *Nucleic Acid Res.* 10, 4731-4751
- Stauber, G.B., et al., 1988, *J.Biolog.Chem.* 35, Vol.263, 19098-19104
- Stauber, G.B., et al., 1989, *J.Biolog.Chem.* 6, Vol.264, 3573-3576
- Torti, F.M. et al., 1985; *Nature* 229:867-869
- Tracey, K.J., et al., 1987, *Nature* 330, 662-666
- Tracey, K.J., et al., 1986, *Science* 234, 470-474
- Urlaub und Chasin, 1980, *Proc.Natl.Acad.Sci.* 77, 4216-4220
- Waage, A., et al., 1987, *Lancet.* ii, 355-357
- Wong, G.H.W., et al., 1986, *Nature* 323, 819-822

## Patent Claims

1. DNA coding for a TNF receptor or fragments thereof, characterized in that it has the formula

```

ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCA
CTG GTG CTC CTG GAG CTG TTG GTG GGA ATA TAC CCC
TCA GGG GTT ATT GGA CTG GTC CCT CAC CTA GGG GAC
AGG GAG AAG AGA GAT AGT GTG TGT CCC CAA GGA AAA
TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC
AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT
CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT
GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC
AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG GAA
ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC
CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC
CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC
AAT TGC AGC CTC TGC CTC AAT GGG ACC GTG CAC CTC
TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC
CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC
TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG
AAG TTG TGC CTA CCC CAG ATT GAG AAT GTT AAG GGC
ACT GAG GAC TCA GGC ACC ACA GTG CTG TTG CCC CTG
GTC ATT TTC TTT GGT CTT TGC CTT TTA TCC CTC CTC
TTC ATT GGT TTA ATG TAT CGC TAC CAA CGG TGG AAG
TCC AAG CTC TAC TCC ATT GTT TGT GGG AAA TCG ACA
CCT GAA AAA GAG GGG GAG CTT GAA GGA ACT ACT ACT
AAG CCC CTG GCC CCA AAC CCA AGC TTC AGT CCC ACT
CCA GGC TTC ACC CCC ACC CTG GGC TTC AGT CCC GTG
CCC AGT TCC ACC TTC ACC TCC AGC TCC ACC TAT ACC
CCC GGT GAC TGT CCC AAC TTT GCG GCT CCC CGC AGA
GAG GTG GCA CCA CCC TAT CAG GGG GCT GAC CCC ATC
CTT GCG ACA GCC CTC GCC TCC GAC CCC ATC CCC AAC
CCC CTT CAG AAG TGG GAG GAC AGC GCC CAC AAG CCA
CAG AGC CTA GAC ACT GAT GAC CCC GCG ACG CTG TAG

```

GCC GTG GTG GAG AAC GTG CCC CCG TTG CGC TGG AAG  
 GAA TTC GTG CGG CGC CTA GGG CTG AGC GAC CAC GAG  
 ATC GAT CGG CTG GAG CTG CAG AAC GGG CGC TGC CTG  
 CGC GAG GCG CAA TAC AGC ATG CTG GCG ACC TGG AGG  
 CGG CGC ACG CCG CGG CGC GAG GCC ACG CTG GAG CTG  
 CTG GGA CGC GTG CTC CGC GAC ATG GAC CTG CTG GGC  
 TGC CTG GAG GAC ATC GAG GAG GCG CTT TGC GGC CCC  
 GCC GCC CTC CCG CCC GCG CCC AGT CTT CTC AGA TGA

or fragments thereof, including degenerate variants thereof.

2. DNA according to claim 1, coding for a secretable TNF-binding protein, characterized in that it has the formula

R<sup>2</sup> GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC  
 CCT CAA AAT AAT TCG ATT TGC TGT ACC AAG TGC CAC  
 AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG  
 GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC  
 TCC TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC  
 CTC AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG  
 GTG GAG ATC TCT TCT TGC ACA GTG GAC CGG GAC ACC  
 GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT  
 TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC  
 CTC TGC CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG  
 GAG AAA CAG AAC ACC GTG TGC ACC TGC CAT GCA GGT  
 TTC TTT CTA AGA GAA AAC GAG TGT GTC TCC TGT AGT  
 AAC TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG TGC  
 CTA CCC CAG ATT GAG AAT

wherein  $R^2$  is optionally absent or represents DNA coding for a polypeptide which can be cleaved in vivo, including degenerate variants thereof.

3. DNA according to claim 2, coding for secretable TNF-binding protein, characterized in that it has the formula defined in claim 2, wherein  $R^2$  represents DNA which codes entirely or partly for a signal sequence.
4. DNA according to claim 2, characterized in that  $R^2$  has the formula CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA.
5. DNA according to claim 3, characterized in that  $R^2$  represents  $R^3$  CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA, wherein  $R^3$  represents DNA coding for a signal peptide.
6. DNA according to claim 5, characterized in that  $R^3$  represents  
 ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCA  
 CTG GTG CTC CTG GAG CTG TTG GTG GGA ATA TAC CCC  
 TCA GGG GTT ATT GGA.
7. Nucleic acids, characterized in that they hybridize with the DNA defined in claim 1 or fragments thereof under conditions of low stringency and code for a polypeptide having the ability to bind TNF or

contain the sequence which codes for such a polypeptide.

8. A recombinant DNA molecule, characterized in that it contains the DNA sequence defined in claim 1, or a degenerate variant or at least a fragment thereof.
9. The recombinant DNA molecule according to claim 8, which is replicable in prokaryotic or eukaryotic host organisms, characterized in that said DNA molecule contains expression control sequences functionally linked to the DNA sequence defined in claim 2, or a degenerate variant or at least a fragment thereof.
10. The recombinant DNA molecule according to claim 9, which is replicable in mammalian cells.
11. The recombinant DNA molecule according to claim 9, characterized in that it contains a DNA defined in any of claims 2-6.
12. The recombinant DNA molecule according to claim 10, characterized in that it contains the DNA defined in claim 6.
13. The recombinant DNA molecule according to claim 12 designated pADTNF-BP.
14. The recombinant DNA molecule according to claim 12 designated pADBTNF-BP.

15. A recombinant DNA molecule, replicable in prokaryotic or eukaryotic host organisms, characterized in that it contains an optionally modified DNA defined in claim 1 or at least a fragment thereof.
16. The recombinant DNA molecule according to claim 15, replicable in mammalian cells.
17. The recombinant DNA molecule according to claim 16 designated pADTNF-R.
18. The recombinant DNA molecule according to claim 16 designated pADBTNF-R.
19. A host organism transformed with at least one of the recombinant DNA molecules defined in claims 11-14.
20. The host organism transformed with a recombinant DNA molecule defined in claims 15-18.
21. A recombinant polypeptide, characterized in that it is encoded by a DNA defined in claim 1.
22. The polypeptide according to claim 21, characterized in that it is the TNF receptor of formula

met gly leu ser thr val pro asp leu leu leu pro  
 leu val leu leu glu leu leu val gly ile tyr pro  
 ser gly val ile gly leu val pro his leu gly asp  
 arg glu lys arg asp ser val cys pro gln gly lys  
 tyr ile his pro gln asn asn ser ile cys cys thr  
 lys cys his lys gly thr tyr leu tyr asn asp cys  
 pro gly pro gly gln asp thr asp cys arg glu cys  
 glu ser gly ser phe thr ala ser glu asn his leu  
 arg his cys leu ser cys ser lys cys arg lys glu  
 met gly gln val glu ile ser ser cys thr val asp  
 arg asp thr val cys gly cys arg lys asn gln tyr  
 arg his tyr trp ser glu asn leu phe gln cys phe  
 asn cys ser leu cys leu asn gly thr val his leu  
 ser cys gln glu lys gln asn thr val cys thr cys  
 his ala gly phe phe leu arg glu asn glu cys val  
 ser cys ser asn cys lys lys ser leu glu cys thr  
 lys leu cys leu pro gln ile glu asn val lys gly  
 thr glu asp ser gly thr thr val leu leu pro leu  
 val ile phe phe gly leu cys leu leu ser leu leu  
 phe ile gly leu met tyr arg tyr gln arg trp lys  
 ser lys leu tyr ser ile val cys gly lys ser thr  
 pro glu lys glu gly glu leu glu gly thr thr thr  
 lys pro leu ala pro asn pro ser phe ser pro thr  
 pro gly phe thr pro thr leu gly phe ser pro val  
 pro ser ser thr phe thr ser ser ser thr tyr thr  
 pro gly asp cys pro asn phe ala ala pro arg arg  
 glu val ala pro pro tyr gln gly ala asp pro ile  
 leu ala thr ala leu ala ser asp pro ile pro asn  
 pro leu gln lys trp glu asp ser ala his lys pro  
 gln ser leu asp thr asp asp pro ala thr leu tyr  
 ala val val glu asn val pro pro leu arg trp lys  
 glu phe val arg arg leu gly leu ser asp his glu  
 ile asp arg leu glu leu gln asn gly arg cys leu  
 arg glu ala gln tyr ser met leu ala thr trp arg  
 arg arg thr pro arg arg glu ala thr leu glu leu  
 leu gly arg val leu arg asp met asp leu leu gly  
 cys leu glu asp ile glu glu ala leu cys gly pro  
 ala ala leu pro pro ala pro ser leu leu arg

or a polypeptide consisting of at least a fragment thereof.

23. The polypeptide according to claim 22, characterized in that it is a TNF binding protein of formula

asp ser val cys pro gln gly lys tyr ile his pro  
 gln asn asn ser ile cys cys thr lys cys his lys  
 gly thr tyr leu tyr asn asp cys pro gly pro gly  
 gln asp thr asp cys arg glu cys glu ser gly ser  
 phe thr ala ser glu asn his leu arg his cys leu  
 ser cys ser lys cys arg lys glu met gly gln val  
 glu ile ser ser cys thr val asp arg asp thr val  
 cys gly cys arg lys asn gln tyr arg his tyr trp  
 ser glu asn leu phe gln cys phe asn cys ser leu  
 cys leu asn gly thr val his leu ser cys gln glu  
 lys gln asn thr val cys thr cys his ala gly phe  
 phe leu arg glu asn glu cys val ser cys ser asn  
 cys lys lys ser leu glu cys thr lys leu cys leu  
 pro gln ile glu asn

or a functional fragment or derivative having the ability to bind TNF.

24. A method for producing a polypeptide according to claim 23, characterized in that a host organism defined in claim 19 is grown and the protein expressed is isolated.
25. A method for producing a recombinant TNF receptor or fragments or modifications thereof, characterized in that a host organism defined in claim 20 is grown and the protein expressed is isolated.
26. Use of a polypeptide defined in claims 21 and 22 for assaying substances for their interaction with said polypeptide and/or with TNF- $\alpha$  and/or with TNF- $\beta$  and/or for their impact on the biological activity of TNF- $\alpha$  and/or TNF- $\beta$ .
27. Use of a host organism defined in claim 20 for assaying substances for their interaction with the TNF receptor and/or their impact on the biological activity of TNF- $\alpha$  and/or TNF- $\beta$ .
28. Use of a polypeptide according to claim 23 for the prophylactic or therapeutic treatment of the human body at indications involving a detrimental effect of TNF- $\alpha$  and/or TNF- $\beta$ .
29. Use of a polypeptide according to claim 23 as a diagnostic agent for detection of TNF- $\alpha$  and/or TNF- $\beta$ .

30. A pharmaceutical composition, characterized in that it contains a polypeptide defined in claim 23 as the therapeutically active component in an amount which is effective to inhibit the biological activity of TNF- $\alpha$  and/or TNF- $\beta$ .