MOLECULAR AND CELLULAR BIOLOGY, June 1991, p. 3020–3026
0270-7306/91/063020-07$02.00/0

Vol. 11, No. 6

Molecular Cloning and Expression of the Type 1 and Type 2 Murine Receptors for Tumor Necrosis Factor

RAYMOND G. GOODWIN,[1]* DIRK ANDERSON,[1] RITA JERZY,[1] TERRI DAVIS,[1] CAMILYNN I. BRANNAN,[2] NEAL G. COPELAND,[2] NANCY A. JENKINS,[2] AND CRAIG A. SMITH[1]

Immunex Corporation, 51 University Street, Seattle, Washington 98101,[1] and Mammalian Genetics Laboratory, ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702[2]

Received 23 January 1991/Accepted 7 March 1991

Clones encoding the type 1 (p80) and type 2 (p60) forms of the murine receptors for tumor necrosis factor (TNF) were isolated by cross-hybridization using probes derived from the cloned human TNF receptors. Each of the murine receptors shows strong sequence homology to the corresponding human receptor (~65% amino acid identity) throughout the molecule but only modest homology, limited to ligand-binding domains, between themselves. The ligand-binding characteristics of the recombinant murine receptors mirror those of the human homologs: both receptor types bind TNF-α and -β with multiple affinity classes, and the ligands cross-compete. Analysis of the murine transcripts encoding these receptors revealed the presence of RNAs for one or both forms of the receptors in all cells examined. It was also demonstrated that for both types of human TNF receptor, variably sized transcripts are observed in different cells. The murine cDNAs were further used to determine the chromosomal locations of the TNF receptor genes. They are not linked, in contrast to the ligands, and map to chromosomes 4 (type 1) and 6 (type 2).

Tumor necrosis factor α (TNF-α) and β (TNF-β, lymphotoxin) are structurally and functionally homologous cytokines which mediate widely pleiotropic biological activities (1). These include cytotoxic effects against tumor cells (5), activation of neutrophils (30), and transcriptional suppression in adipose tissue of key enzymes regulating lipid homeostasis (1). TNF also induces the secretion of interleukin-1 and is a primary mediator of inflammation and endotoxin-induced shock (2). These TNF-induced activities are transduced by cell surface receptors present on a wide variety of cell types (1). The development of non-cross-reacting monoclonal antibodies to these receptors and the purification of two forms of soluble TNF-binding proteins (11, 25, 29) suggested the existence of two distinct human TNF receptors (3, 14). Both forms of the human TNF receptors have now been cloned (18, 27, 31) and shown to have similar extracellular, ligand-binding domains which define a new superfamily of receptors (31). The recombinant human receptors appear to bind both TNF-α and TNF-β with similar high affinities (27, 31).

Here we report the isolation and expression of cDNAs encoding the homologous murine TNF (muTNF) receptors. As found for the human receptors, the murine type 1 (p80, TNFR-1) and 2 (p60, TNFR-2) receptors show only modest and restricted sequence homology to each other. However, the murine forms show strong homology to their human analogs and display similar ligand-binding characteristics. The availability of cloned murine TNF receptors will aid in the continuing study of the mechanisms by which ligand-receptor interactions in the TNF system elicit such a wide variety of cellular responses.

MATERIALS AND METHODS

Synthesis and screening of a cDNA library. Synthesis of the cDNA library utilizing RNA derived from the antigen-dependent murine helper T-cell line 7B9 has been described (21). Briefly, oligo(dT)-primed cDNA was cloned into λZAP (Stratagene, San Diego, Calif.), using *Eco*RI linkers. The amplified library was plated as instructed by the manufacturer, and duplicate plaque lifts were screened with radiolabeled human TNF receptor cDNA inserts. A human TNFR-2 cDNA was isolated from a human placental library by using synthetic oligonucleotide probes based on published amino acid sequence data of a TNF-binding protein (25). Conditions of hybridization were as previously described (21) except that the temperature was lowered to 37°C. The filters were washed in 2× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at either 55°C (TNFR-1) or 63°C (TNFR-2).

RNA blot analysis. Total cellular RNA was isolated by a guanidinium hydrochloride procedure (19). For Northern (RNA) analysis, the RNA was fractionated on a 1.1% agarose-formaldehyde gel and blotted onto Hybond (Amersham) as recommended by the manufacturer. The blots were probed with antisense riboprobes as previously described (31). The entire cDNA inserts were used as templates in the synthesis of probes for murine and human TNFR-2. Synthesis of an antisense probe for the human TNFR-1 utilized a 630-bp *Not*I-*Bgl*II fragment, while synthesis of a probe for the muTNFR-1 utilized a 1,540-bp *Eco*RI-*Bam*HI fragment which encompassed the entire coding region.

Interspecific backcross mapping. Interspecific backcross progeny were generated by mating (C57BL/6J × *Mus spretus*)F_1 females and C57BL/6J males as previously described (4). A total of 205 N_2 progeny were obtained; a random subset of these N_2 mice was used to map the TNFR-1 and TNFR-2 loci (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described previously (16). All blots were prepared with Zetabind nylon membrane (AMF-Cuno). The TNFR-1 probe was a 1,540-bp *Eco*RI-*Bam*HI fragment from the cloned

* Corresponding author.

mouse cDNA; the TNFR-2 probe was a 1.9-kb mouse cDNA. The probes were labeled with [α-^{32}P]dCTP by using a nick translation labeling kit (Boehringer Mannheim). Washing was done to final stringencies of 0.1× SSCP, 0.1% sodium dodecyl sulfate (SDS), and 65°C (TNFR-1) and 0.2× SSCP, 0.1% SDS, and 65°C (TNFR-2). The TNFR-1 probe detected 7.3- and 3.6-kb fragments in *Bgl*I-digested C57BL/6J DNA and an 11.2-kb fragment in *Bgl*I-digested *M. spretus* DNA. The TNFR-2 probe detected a 2.2-kb *Taq*I fragment in C57BL/6J DNA and 3.6- and 1.0-kb fragments in *Taq*I-digested *M. spretus* DNA. The probes and restriction fragment length polymorphisms (RFLPs) for loci linked to TNFR-1, including the lymphocyte-specific protein tyrosine kinase (*Lck*), Gardner-Rasheed feline sarcoma viral oncogene homolog (*Fgr*), and atrial natriuretic factor (*Anf*) loci, have been described (6). The probe for the Sloan-Kettering viral oncogene homolog (*Ski*) was a 1.1-kb human cDNA (23) that detected 4.6-, 3.3-, and 1.8-kb fragments in *Taq*I-digested C57BL/6J DNA and 6.0- and 1.8-kb fragments in *Taq*I-digested *M. spretus* DNA. The 6.0-kb *M. spretus*-specific *Taq*I fragment was monitored in these studies. The probes and RFLPs for two loci linked to TNFR-2, the *ras*-related fibrosarcoma oncogene homolog 1 (*Raf-1*) and fibroblast growth factor 6 (*Fgf-6*) loci, have been described (9). The probe for lymphocyte antigen 4 (*Ly-4*) was a 1.3-kb mouse cDNA (33) that detected 4.5-, 2.5-, and 1.5-kb fragments in *Pvu*II-digested C57BL/6J DNA and 2.5-, 1.5-, 1.0-kb fragments in *Pvu*II-digested *M. spretus* DNA. The 1.0-kb *M. spretus*-specific fragment was monitored in these studies. Recombination distances were calculated as described previously (13), using the computer program SPRETUS MADNESS developed by D. Dave (Data Management Services, Inc., Frederick, Md.) and A. M. Buchberg (NCI-Frederick Cancer Research and Development Center, ABL-Basic Research Program, Frederick, Md.). Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

Transfections, cytokines, and equilibrium binding assays. Human TNF-α was expressed in yeast cells, using the α-factor secretion system, as a fusion polypeptide containing the mature sequence (26) but incorporating a hydrophilic octapeptide at the N terminus to aid in purification (15). The material possessed a specific biological activity of 3×10^8 U/mg in a standard cytolytic assay (32) and gel filtered at 55 kDa, consistent with a trimeric status (17). Human TNF-β was purchased from R&D Sciences (Minneapolis, Minn.). The specific biological activity was 1×10^8 U/mg. Purified muTNF-α, expressed in *Escherichia coli*, was a gift of Ed Amento (Genentech, South San Francisco, Calif.). All ligands were radioiodinated with IODOGEN (Pierce) to a specific activity of 10^{15} cpm/mmol (31).

For quantitative in situ binding studies, COS cell monolayers in six-well trays (Costar) were transfected with the mammalian expression vector pDC302 containing the TNF receptor cDNAs and cultured for 3 days (31). For direct binding studies, the monolayers were washed once with phosphate-buffered saline (PBS) and then incubated with 1.0 ml of ^{125}I-TNF at various concentrations in binding medium (RPMI 1640, 4% bovine serum albumin, 0.1% sodium azide, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4]) at 4°C for 4 h. Free ^{125}I-TNF was determined by counting gamma emissions in the supernatant. Monolayers were then washed once with ice-cold RPMI, detached with 0.1% trypsin in PBS, and counted to determine bound ligand. Nonspecific ligand binding was determined by inclusion of a 200-fold molar excess of unlabeled ligand. Inhibition assays used ^{125}I-TNF-α at 0.4 nM in the presence of serially diluted unlabeled ligand. Data were analyzed by using RS/1 (BBN Software Products Corp., Cambridge, Mass.), a commercially available scientific data processing package running on a VAX 11/750 under the VMS operating system. Equilibrium binding data were analyzed with sums of simple Michaelis-Menten terms as described previously (10); inhibition data were analyzed with an equation for competitive inhibition between two ligands for two types of sites (8).

Nucleotide sequence accession number. The nucleotide sequences of the cloned muTNF receptor cDNAs have been submitted to GenBank. The accession number for TNFR-1 is M59378; that for TNFR-2 is M59377.

RESULTS

Isolation of muTNF receptor cDNAs. Murine cDNAs encoding both forms of TNF receptors were obtained by cross-hybridization with the homologous human cDNAs. The human cDNAs were radiolabeled and used to probe a cDNA library prepared from RNA isolated from a murine T-cell line termed 7B9 (see Materials and Methods). The largest cDNAs were sequenced and shown to contain the entire coding regions for both forms of murine receptors. A comparison of the predicted amino acid sequences of the cloned murine receptors with those of the analogous human receptors is shown in Fig. 1. These alignments demonstrate the high degree of homology which occurs throughout the corresponding forms of the murine and human TNF receptors. Alignment of the murine and human TNFR-1 (Fig. 1A) and TNFR-2 (Fig. 1B) demonstrated 64 and 65% amino acid identity, respectively. In contrast, homology between the two forms of murine receptors is, as with the human forms (18, 27, 31), confined to the extracellular, ligand-binding domains (28% identity). As shown in Fig. 1, all of the cysteine residues in the extracellular and transmembrane domains are conserved between the murine and human TNFR-1 and TNFR-2. In the cytoplasmic domains of TNFR-2, three of the five cysteines are conserved; in TNFR-1, five cysteines are conserved, with one additional cysteine present in the murine receptor cytoplasmic domain. Conspicuously conserved in the cytoplasmic domain of TNFR-1 are six contiguous serine residues (underlined in Fig. 1A), a feature absent in TNFR-2. Both forms of the receptor also contain consensus sequences for potential phosphorylation sites by protein kinase C (Ser/Thr-X-Arg/Lys; 34). In both receptor types, only one of these sites is conserved in both the human and murine receptors (double underline in Fig. 1).

Binding characteristics. The equilibrium ligand-binding characteristics of recombinant muTNFR-1 and muTNFR-2, expressed in COS cells, are shown in Fig. 2. Scatchard analysis clearly shows that both receptors exhibit high-affinity binding sites for ^{125}I-muTNF-α: K_a (TNFR-1) = 6.0 ± 2.9 nM^{-1}; K_a (TNFR-2) = 2.3 ± 0.18 nM^{-1} (Fig. 2A to D). TNFR-1 also exhibits a low-affinity class (K_a = 0.13 ± 0.03 nM^{-1}; Fig. 2B). In contrast, the Scatchard plots of TNFR-2 do not unambiguously reveal this low-affinity site at similar ligand concentrations (up to 5 nM). Both receptors, however, display complex inhibition behavior with ^{125}I-muTNF-α (Fig. 2E and F) that is well described by a two-site competitive inhibition model (8). TNFR-1 shows low-affinity (K_{i1} = 1.79 ± 0.041 nM^{-1}) and high-affinity (K_{i2} = 0.21 ± 0.015 pM^{-1}) sites with competition from unlabeled muTNF-α. TNFR-2 also reveals two inhibition classes with

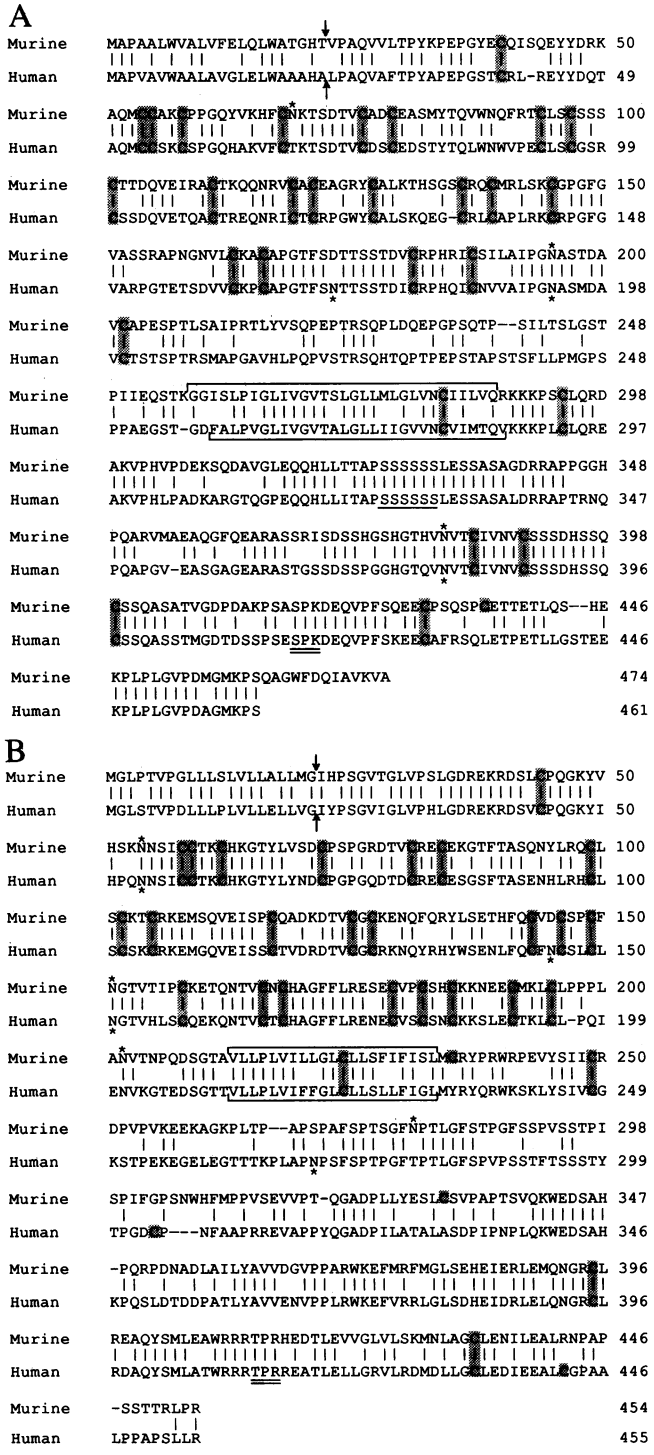

FIG. 1. Alignment of human and murine TNFR-1 (A) and TNFR-2 (B) amino acid sequences. Vertical lines indicate identical amino acids, and horizontal lines mark gaps introduced to provide maximum alignment. Cysteine residues are shaded. The arrows indicate the predicted amino termini of the mature proteins, and potential N-linked glycosylation sites are identified by asterisks. The transmembrane domains are bracketed, the conserved six continuous serine residues in TNFR-1 are singly underlined, and the conserved potential protein kinase C sites are doubly underlined.

heterologous human TNF-β, low (K_{i1} = 1.2 ± 0.05 nM^{-1}) and high (K_{i2} = 0.19 ± 0.09 pM^{-1}). Similar inhibition data have been published for native and recombinant human forms of these receptors (27, 31).

RNA blot analysis. Northern blot analysis of the muTNF receptor transcripts is shown in Fig. 3. Probes specific for muTNFR-1 demonstrated the presence of two size classes of transcripts of approximately 3.2 and 4.1 kb in five of the six cell types examined. This included the mast cell line MC9, the helper T-cell line D10, the pre-B-cell line 70Z/3, the bone marrow stromal cell line +/+, and splenic tissue. In addition, a less abundant transcript of 5.1 kb was present in some of the cells. In contrast, we were unable to detect any transcripts for TNFR-1 in the lymphoma cell line EL4-3$^+$. Analysis of the murine TNFR-2 transcripts revealed a single size class of approximately 2.3 kb (Fig. 3). Transcripts for this receptor were detected in MC9, EL4-3$^+$, +/+ cells, and splenic tissue but not in D10 and 70Z/3 cells. Thus, examples of cells expressing transcripts for either a single type of TNF receptor or both forms were shown to exist.

Northern blot analyses of human TNF receptor transcripts are also shown in Fig. 3. As previously observed (31), there are two size classes of transcripts for human TNFR-1, with some cells expressing a single size class of transcripts and others expressing both. Northern analysis of the human TNFR-2 transcripts demonstrated three size classes of RNA of 2.3, 6.4, and 12 kb (Fig. 3). The monocytic cell line THP-1 was shown to express only the 2.3-kb transcript, while the pre-B-cell line JM-1 expressed exclusively the 6.4-kb transcript. In contrast, the pancreatic tumor line HPT2 expressed both of these size classes of transcripts, while the tonsillar B and T cells demonstrated the presence of an additional transcript of roughly 12 kb. The origin or possible relevance of these different size classes of transcripts is presently unknown.

Chromosomal mapping. The mouse chromosomal locations of the TNF receptor loci were determined by interspecific backcross analysis, using progeny derived from matings of [(C57BL/6J × *M. spretus*)F_1 × C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 660 loci that are well distributed among all autosomes as well as the X chromosome. C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative RFLPs, using the TNF receptor probes described in Materials and Methods. The 11.2-kb *M. spretus*-specific *BglI* RFLP (see Materials and Methods) was used to monitor the segregation of the TNFR-1 locus in backcross mice. The mapping results indicated that TNFR-1 is located in the distal region of mouse chromosome 4 tightly linked to *Anf*. The additional loci included in this analysis are *Lck*, *Fgr*, and *Ski*. Although 107 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 4), up to 122 mice were typed for some markers. Each locus was analyzed in pairwise combinations for recombination frequencies, using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are centromere-*Lck* (6/122)-*Fgr* (7/110)-(*Tnfr-1* [0/107]-*Anf*) (4/116)-*Ski*. The recombination frequencies (expressed as genetic distances in centimorgans ± standard error) are *Lck* (4.9 ± 2.0)-*Fgr* (6.4 ± 2.3)-(*Tnfr-1*, *Anf*) (3.5 ± 1.7)-*Ski*. No crossovers were detected between TNFR-1 and *Anf*, giving an upper 95% confidence limit of 2.8 centimorgans between the two loci. The placement of these

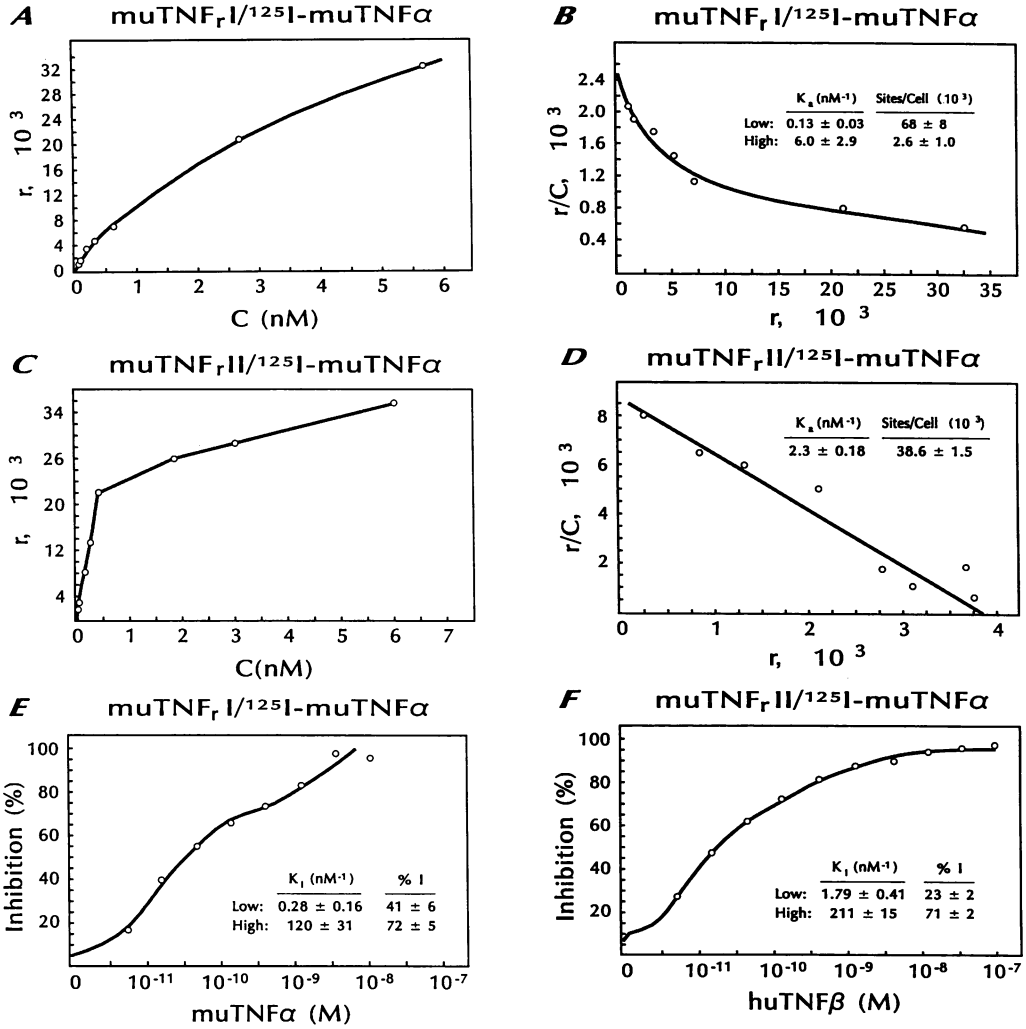

FIG. 2. Ligand-binding characteristics of recombinant murine TNFR-1 and TNFR-2. Assays were performed in situ on COS cells transiently expressing receptors as described in Materials and Methods. (A) Specific binding of ^{125}I-muTNF-α to TNFR-1 after 2 h, 4°C. Data are plotted as bound (r) versus free ligand concentration (C). (B) Replotting of the data in panel A in a Scatchard coordinate system, using nonlinear least-squares analysis of a two-site fit (10). (C) Specific binding of ^{125}I-muTNF-α to TNFR-2 after 2 h, 4°C. (D) Replotting of the data in panel C in a Scatchard coordinate system with linear least-squares analysis. (E) Inhibition of ^{125}I-muTNF-α to TNFR-1 with unlabeled muTNF-α. (F) Inhibition of ^{125}I-muTNF-α to TNFR-2 with unlabeled human TNF-β (huTNFβ). Data in panels E and F were fitted by using a curve describing simple competitive inhibition at two sites (8).

loci relative to other chromosome 4 markers and a comparison of the interspecific backcross map with the composite intraspecific backcross map have been reported previously (6). Finally, the distal region of mouse chromosome 4 shares a region of homology with human chromosome 1. In particular, *Anf* has been placed at 1p36 (summarized in reference 6). The tight linkage between TNFR-1 and *Anf* suggests that the TNFR-1 locus may reside on human chromosome 1p36 as well.

The TNFR-2 locus was mapped by following the segregation of the 3.6-kb *M. spretus*-specific *Taq*I RFLP (see Materials and Methods). The mapping results indicated that TNFR-2 is located in the distal region of mouse chromosome 6 tightly linked to *Raf-1*, *Ly-4*, and *Fgf-6*. Although 155 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 5), up to 188 mice were typed for some markers. Again, each locus was analyzed in pairwise combinations for recombination frequencies, using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are centromere-*Raf-1* (6/162)-(*Tnfr-2* [0/172]-*Ly-4*) (3/188)-*Fgf-6*. The recombination frequencies (expressed as genetic distances in centimorgans ± standard error) are *Raf-1* (3.7 ± 1.5)-(*Tnfr-2*, *Ly-4*) (1.6 ± 0.9)-*Fgf-6*. No crossovers were detected between TNFR-2 and *Ly-4*, giving an upper 95% confidence limit of 1.6 centimorgans between the two loci. The placement of these loci relative to other chromosome 6 markers and a comparison of the interspecific backcross map with the composite intraspecific backcross map have been reported previously (9). The tight linkage between TNFR-2 and *Ly-4*, which is located on human chromosome 12p12-pter, suggests that TNFR-2 may also be located on the short arm of human chromosome 12.

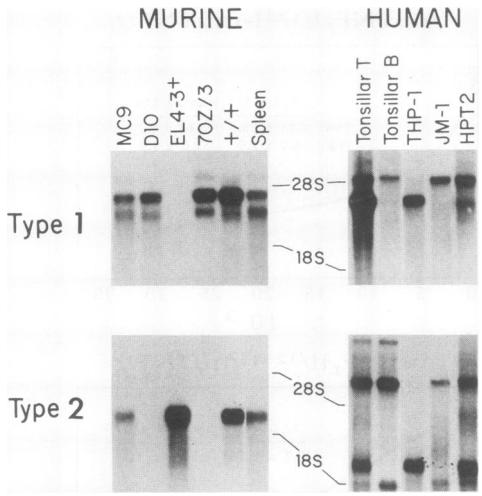

FIG. 3. RNA blot analysis of TNF receptor mRNA. Polyadenylated RNA (3 μg) was used from each source except for tonsillar B cells (5 μg of total RNA). Variable exposure times were used for the murine type 2 blot.

DISCUSSION

Here we report the isolation and characterization of cDNAs encoding both forms of the murine receptors for TNF. Like the human receptors, muTNFR-1 and -2 share only modest sequence homology (28% identity) that is restricted to the cysteine-rich, extracellular region. No similarity exists between the corresponding leader, transmembrane, or cytoplasmic domains. Conservation is therefore evident only in framework residues which comprise the ligand-binding domain and specify the global architecture of this emerging superfamily of integral membrane proteins. In contrast, analogous receptors between species show strong homology (65% identity) along the entire sequence. Thus, duplication and divergence of the ancestral gene encoding the proto-TNF receptor must considerably predate speciation. In fact, quantitative sequence alignments between all 12 presently known members of this superfamily reveal that TNFR-1 and TNFR-2 (murine or human) are among the least similar pairs (30a). Therefore, the TNF receptor genes are presumably very ancient.

The cytoplasmic domains of the TNF receptors show no homology to the catalytic domains of protein kinases, and thus the role of conserved cytoplasmic sequences in signal transduction is unknown. Of particular note is the conservation of six contiguous serine residues in the cytoplasmic domain of TNFR-1. Serine/threonine-rich cytoplasmic domains have been observed in a number of other cytokine receptors and have been suggested to be sites of specific phosphorylation involved in signal transduction (21). In addition, both forms of the receptors contain a potential protein kinase C phosphorylation site which has been conserved between species. Previous work has shown that activators of protein kinase C effect down-modulation of receptor levels (28). Construction of deletion mutants in these regions may help to define the role of these domains in signal transduction.

The equilibrium ligand-binding characteristics of recombinant muTNFR-1 and -2 are complex and appear similar to those published for the corresponding human forms. MuTNFR-1, like its human homolog (31), displays curvilinear Scatchard plots with homologous ^{125}I-TNF-α consistent

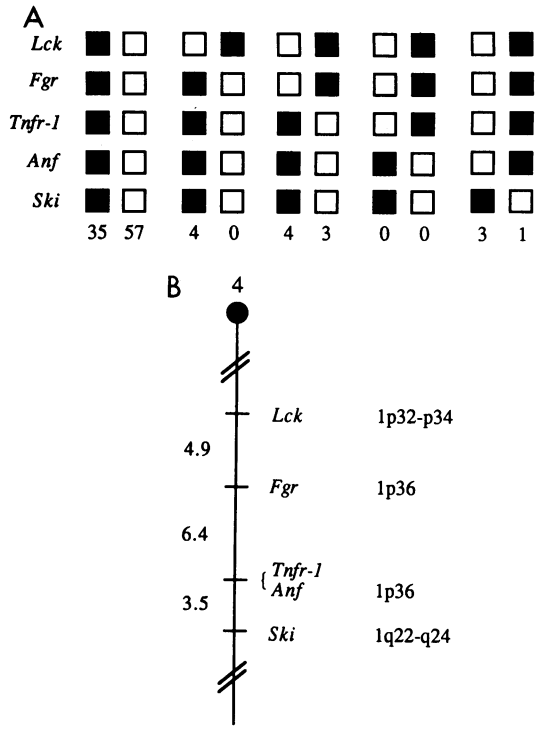

FIG. 4. Position of the TNFR-1 locus on mouse chromosome 4, placed by interspecific backcross analysis. (A) Segregation patterns of TNFR-1 and flanking genes in 107 backcross animals that were typed in common for this receptor gene. For individual pairs of loci, more than 107 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*)F_1 parent. The shaded boxes represent the presence of a C57BL/6J allele, and white boxes represent the presence of an *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. (B) Partial chromosome 4 linkage map showing the location of TNFR-1 in relation to linked genes. Recombination distances between loci in centimorgans are shown to the left of the chromosome, and the positions of all loci except TNFR-1 in human chromosomes (6, 7) are shown to the right.

with the presence of at least two affinity classes, low (K_{a1} = 0.13 ± 0.03 nM^{-1}) and high (K_{a2} = 6.0 ± 2.9 nM^{-1}). The numerical values of these affinities are, in fact, similar to the corresponding affinities of human TNFR-1 for human TNF-α (31). Scatchard analysis of muTNFR-2 binding of muTNF-α, however, usually reveals only a single class of high-affinity sites (K_a = 2.3 ± 0.18 nM^{-1}), in accord with results of Loetscher et al. (18) using recombinant human TNFR-2 and human TNF-α. Schall et al. (27), however, detect two affinity classes with recombinant human TNFR-2 and human TNF-α. We have been unable to express full-length forms of murine (or human) TNFR-2 in COS cells at sufficiently high levels for accurate binding analysis and have therefore used a membrane-bound but cytoplasmically truncated form. Whether this affects extracellular ligand binding is unclear. However, inhibition data of ^{125}I-muTNF-α binding to the recombinant murine receptors also demonstrate complex binding behavior (Fig. 2E and F). Competition with muTNF-α (muTNFR-1) or human TNF-β (muTNFR-2) reveals two affinity classes: K_i (low, TNFR-1) = 0.27 ± 0.16 nM^{-1}; K_i (high, TNFR-1) = 0.12 ± 0.03 pM^{-1}); K_i (low, TNFR-2) = 1.8 ± 0.41 nM^{-1}, K_i (high, TNFR-2) = 0.21 ±

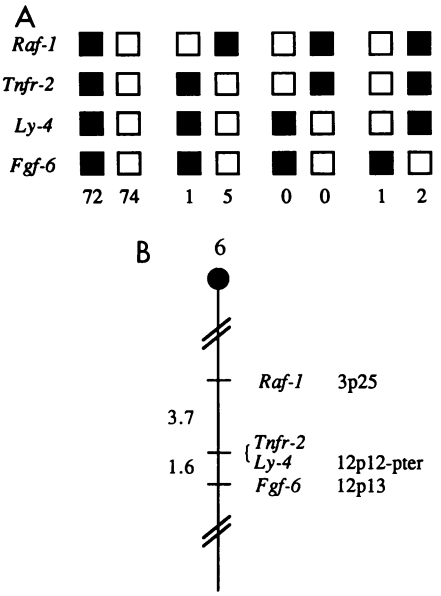

FIG. 5. Position of the TNFR-2 locus on mouse chromosome 6 placed by interspecific backcross analysis as described in the legend to Fig. 4. (A) Segregation patterns of TNFR-2 and flanking genes in 155 backcross animals that were typed in common for TNFR-2. (B) Partial chromosome 6 linkage map showing the location of TNFR-2 in relation to linked genes. Recombination distances between loci in centimorgans are shown to the left of the chromosome, and the positions of all loci except TNFR-2 in human chromosomes (reviewed in reference 9) are shown to the right.

0.015 pM^{-1}. Thus, both inhibition and direct binding data suggest a common lower-affinity class of sites, while inhibition data suggest an additional very high affinity site of ~10 pM^{-1}. Since TNF-α is predominantly a homotrimer (17), these results suggest the existence of at least two and perhaps three distinct affinity classes, corresponding to mono-, di-, and trivalent ligand binding to receptors free to diffuse in the plane of the membrane (31). The fractional occupancy of each class should thus be a complex function of relative affinities as well as receptor and ligand densities. Some cell types also appear to express both types of receptors (3; Fig. 3), raising the possibility of heterologous receptor-ligand complexes. A more comprehensive analysis of the kinetic and equilibrium ligand-binding characteristics of this system is needed.

We also reported here the chromosomal locations of the genes for the muTNF receptors. In contrast to the ligands (22), the murine genes are not linked and do not map to the major histocompatibility complex locus. The genes for TNFR-1 and TNFR-2 map to the distal regions of chromosomes 4 and 6, respectively. It is interesting to note that TNFR-2 maps near the crooked locus (20), which arose spontaneously and is associated with pleiotropic effects, including abnormal bone development. In this regard, we have recently observed that TNFR-2 transcripts are expressed early in the development of the mouse embryo (day 8), at which time transcripts for TNFR-1 are not detectable (12a). Thus, it will be interesting to characterize the developmental expression of TNFR-2 and see if it is a candidate for this mutation.

Because both receptors are encoded by single-copy genes, the variably sized transcripts that are observed by Northern analysis are likely due to alternative processing of the original transcripts. We currently have no evidence to indicate alternative splicing occurs within either murine gene. Thus, it is possible that the use of alternative polyadenylation sites results in the different-size transcripts that we observed. Alternatively sized transcripts are also observed for both forms of the human TNF receptors, which has previously been described for TNFR-1 (31). However, in previous reports on TNFR-2, only a single size class of transcripts was observed (18, 27). Here we have demonstrated that different-size transcripts for human TNFR-2 are in fact produced in different cell types, the significance of which is unknown at present. Previous reports have described the isolation of soluble forms of the TNF receptors in human urine and serum (11, 25, 27, 29). There is presently no evidence that these soluble TNF receptors are encoded by differentially spliced transcripts, as has been observed for several cytokine receptors (12, 21). Thus, the soluble TNF receptors appear to arise from the proteolytic shedding of the cell surface receptors. This conclusion is consistent with the observation that CHO cells transfected with a TNF receptor cDNA produced both cell surface and soluble forms of the receptor (24).

Future experiments will examine the role of the individual receptors (or ligands) eliciting specific biological responses. For example, transgenic mice may be created in which either TNFR-1 or TNFR-2 is specifically deleted and the biological consequences are assessed. Because neither receptor contains structural features common to protein kinases or any other catalytic functions, future studies will also investigate the possible presence of receptor-associated peptides required for signal transduction.

ACKNOWLEDGMENTS

We thank Nobuo Nomura and Beverly Mock for probes, K. Shanebeck, L. Solam, D. J. Gilbert, D. A. Swing, and B. Cho for excellent technical assistance, and Debbie Wright for manuscript preparation.

This research was supported in part by the National Cancer Institute under contract NO1-CO-74101 with ABL.

ADDENDUM IN PROOF

We have also shown that human TNF cross-reacts only with muTNFR-2 and not with muTNFR-1.

REFERENCES

1. **Beutler, B., and A. Cerami.** 1988. Tumor necrosis, cachexia, shock and inflammation: a common mediator. Annu. Rev. Biochem. **57**:505–518.
2. **Beutler, B., and A. Cerami.** 1989. The biology of cachectin/TNF—a primary mediator of the host response. Annu. Rev. Immunol. **7**:625–655.
3. **Brockhaus, M., H.-J. Schoenfeld, E.-J. Schlaeger, W. Hunziker, W. Lesslauer, and H. Loetscher.** 1990. Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. Proc. Natl. Acad. Sci. USA **87**:3127–3131.
4. **Buchberg, A. M., H. G. Bedigian, B. A. Taylor, E. Brownell, J. N. Ihle, S. Nagata, N. A. Jenkins, and N. G. Copeland.** 1988. Localization of *Evi-2* to chromosome 11: linkage to other proto-oncogene and growth factor loci using interspecific backcross mice. Oncogene Res. **2**:149–165.
5. **Carswell, E. A., L. J. Old, R. L. Kassel, S. Green, N. Fiore, and B. Williamson.** 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. USA **72**:3666–3670.
6. **Ceci, J. D., L. D. Siracusa, N. A. Jenkins, and N. G. Copeland.** 1989. A molecular genetic linkage map of mouse chromosome 4

including the localization of several proto-oncogenes. Genomics **5**:699–709.

7. **Chaganti, R. S. K., I. Balazs, S. C. Jhanwar, V. V. V. S. Murty, P. R. K. Koduru, K.-H. Grzeschik, and E. Stavnerzer.** 1986. The cellular homologue of the transforming gene of SKV avian retrovirus maps to human chromosome region lq22→q24. Cytogenet. Cell Genet. **43**:181–186.
8. **Cuatrecasas, P., and M. Hollenberg.** 1975. Membrane receptors and hormone action. Adv. Protein Chem. **30**:251–429.
9. **de Lapeyriere, O., O. Rosnet, D. Benharroch, F. Raybaud, S. Marchetto, J. Planche, F. Galland, M.-G. Mattei, N. G. Copeland, N. A. Jenkins, F. Coulier, and D. Birnbaum.** 1990. Structure, chromosome mapping and expression of the murine *Fgf-6* gene. Oncogene **5**:823–831.
10. **Dower, S. K., K. Ozato, and D. M. Segal.** 1984. The interaction of monoclonal antibodies with MHC class I antigens on mouse spleen cells. I. Analysis of the mechanism of binding. J. Immunol. **132**:751–758.
11. **Engelmann, H., D. Novick, and D. Wallach.** 1990. Two tumor necrosis factor-binding proteins purified from human urine. Evidence for immunological cross-reactivity with cell surface tumor necrosis factor receptors. J. Biol. Chem. **265**:1531–1536.
12. **Goodwin, R. G., D. Friend, S. F. Ziegler, R. Jerzy, B. A. Falk, S. Gimpel, D. Cosman, S. K. Dower, C. J. March, A. E. Namen, and L. S. Park.** 1990. Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. Cell **60**:941–951.

12a. **Goodwin, R. G., and C. A. Smith.** Unpublished data.

13. **Green, E. L.** 1981. Linkage, recombination, and mapping, p. 77–113. *In* Genetics and probability in animal breeding experiments. Macmillan, New York.
14. **Hohmann, H.-P., R. Remy, M. Brockhaus, and A. P. G. M. van Loon.** 1989. Two different cell types have different major receptors for human tumor necrosis factor (TNF α). J. Biol. Chem. **264**:14927–14934.
15. **Hopp, T. P., K. S. Prickett, V. L. Price, R. T. Libby, C. J. March, D. P. Cerretti, D. L. Urdal, and P. J. Conlon.** 1988. A short polypeptide marker sequence useful for recombinant protein identification and purification. Biotechnology **6**:1204–1210.
16. **Jenkins, N. A., N. G. Copeland, B. A. Taylor, and B. K. Lee.** 1982. Organization, distribution, and stability of endogenous ecotropic murine leukemia virus DNA sequences in chromosomes of *Mus musculus*. J. Virol. **43**:26–36.
17. **Jones, E. Y., D. I. Stuart, and N. P. C. Walker.** 1989. Structure of tumor necrosis factor. Nature (London) **338**:225–228.
18. **Loetscher, H., Y.-C. E. Pan, H.-W. Lahm, R. Gentz, M. Brockhaus, H. Tabuchi, and W. Lesslauer.** 1990. Molecular cloning and expression of the human 55kd tumor necrosis factor receptor. Cell **61**:351–359.
19. **March, C. J., B. Mosley, A. Larsen, D. P. Cerretti, G. Braedt, V. Price, S. Gillis, C. S. Henney, S. R. Kronheim, K. Grabstein, P. J. Conlon, T. P. Hopp, and D. Cosman.** 1985. Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. Nature (London) **315**:641–646.
20. **Morgan, W. C.** 1954. A new crooked tail mutation involving distinctive pleiotropism. J. Genet. **52**:354–373.
21. **Mosley, B., M. P. Beckmann, C. J. March, R. L. Idzerda, S. D. Gimpel, T. VandenBos, D. Friend, A. Alpert, D. Anderson, J. Jackson, J. M. Wignall, C. Smith, B. Gallis, J. E. Sims, D. Urdal, M. B. Widmer, D. Cosman, and L. S. Park.** 1989. The murine interleukin-4 receptor: molecular cloning and characterization of secreted and membrane bound forms. Cell **59**:335–348.
22. **Nedwin, G. E., S. L. Naylor, A. Y. Sakaguchi, D. Smith, J. Jarrett-Nedwin, D. Pennica, D. V. Goeddel, and P. W. Gray.** 1985. Human lymphotoxin and tumor necrosis factor genes: structure homology and chromosomal localization. Nucleic Acids Res. **13**:6361–6367.
23. **Nomura, N., S. Sasamoto, S. Ishii, T. Date, M. Matsui, and R. Ishizaki.** 1989. Isolation of human cDNA clones of *ski* and the *ski*-related gene, *sno*. Nucleic Acids Res. **17**:5489–5500.
24. **Nophar, Y., O. Kemper, C. Brakebusch, H. Engelmann, R. Zwang, D. Aderka, H. Holtmann, and D. Wallach.** 1990. Soluble forms of tumor necrosis factor receptors (TNF-Rs). The cDNA for the type I TNF-R, cloned using amino acid sequence data of its soluble form, encodes both the cell surface and a soluble form of the receptor. EMBO J. **9**:3269–3278.
25. **Olsson, I., L. Mikael, E. Nilsson, C. Peetre, H. Thysell, A. Grubb, and G. Adolf.** 1989. Isolation and characterization of a tumor necrosis factor binding protein from urine. Eur. J. Haematol. **42**:270–275.
26. **Pennica, D., G. E. Nedwin, J. S. Hayflick, P. H. Seeburg, R. Derynck, M. A. Palladino, W. J. Kohr, B. B. Aggarwal, and D. V. Goeddel.** 1984. Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature (London) **312**:724–729.
27. **Schall, T. J., M. Lewis, K. J. Koller, A. Lee, G. C. Rice, G. H. W. Wong, T. Gatanaga, G. A. Granger, R. Lentz, H. Raab, W. J. Kohr, and D. V. Goeddel.** 1990. Molecular cloning and expression of a receptor for human tumor necrosis factor. Cell **61**:361–370.
28. **Scheurich, P., R. Unglaub, B. Maxeiner, B. Thoma, G. Zugmaier, and K. Pfizenmaier.** 1986. Rapid modulation of tumor necrosis factor membrane receptors by activators of protein kinase C. Biochem. Biophys. Res. Commun. **141**:855–860.
29. **Seckinger, P., S. Isaaz, and J.-M. Dayer.** 1989. Purification and biologic characterization of a specific tumor necrosis factor α inhibitor. J. Biol. Chem. **264**:11966–11973.
30. **Shalaby, M. R., B. B. Aggarwal, E. Rinderknecht, L. P. Svedersky, B. S. Finkle, and M. A. Palladino.** 1985. Activation of polymorphonuclear neutrophil functions by gamma-interferon and tumor necrosis factors. J. Immunol. **135**:2069–2073.

30a. **Smith, C., T. Davis, J. Wignall, W. Din, T. Farrah, C. Upton, G. McFadden, and R. G. Goodwin.** 1991. T2 open reading frame from the Shope fibroma virus encodes a soluble form of the TNF receptor. Biochem. Biophys. Res. Commun. **176**:335–342.

31. **Smith, C. A., T. Davis, D. Anderson, L. Solam, M. P. Beckmann, R. Jerzy, S. K. Dower, D. Cosman, and R. G. Goodwin.** 1990. A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. Science **248**:1019–1023.
32. **Smith, R. A., M. Kirstein, W. Fiers, and C. Baglioni.** 1986. Species specificity of human and murine tumor necrosis factor. A comparative study of tumor necrosis factor receptors. J. Biol. Chem. **261**:14871–14874.
33. **Tourvieille, B., S. D. Gorman, E. H. Field, T. Hunkapiller, and J. R. Parnes.** 1986. Isolation and sequence of L3T4 complementary DNA clones: expression in T cells and brain. Science **234**:610–614.
34. **Woodget, J. R., K. L. Gould, and T. Hunter.** 1986. Substrate specificity of protein kinase C. Eur. J. Biochem. **161**:177–184.