Promoter Structure and Transcriptional Activation of the Murine TSG-14 Gene Encoding a Tumor Necrosis Factor/Interleukin-1-inducible Pentraxin Protein*

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Human TNF-stimulated gene 14 (TSG-14) encodes a secreted 42-kDa glycoprotein that shows significant homology to proteins of the pentraxin family, which includes the acute phase reactants C-reactive protein and serum amyloid P component. Levels of TSG-14 protein (also termed PTX-3) become elevated in the serum of mice and humans after injection with bacterial lipopolysaccharide, but in contrast to conventional acute phase proteins, the bulk of TSG-14 synthesis in the intact organism occurs outside the liver. In the present study we cloned and partially sequenced murine genomic TSG-14 DNA. Analysis of the coding region predicts a high degree of amino acid sequence homology between murine and human TSG-14 (88 and 75% identity in the first and second exons, respectively). The promoter of the TSG-14 gene lacks consensus sequences for either a TATA box or CCAAT box. Primer extension analysis and 5′ nuclelease protection assay revealed one major transcription start site, situated within a consensus sequence for an initiator element. Sequence analysis of a 1.4-kilobase pair fragment of the 5′-flanking region of the TSG-14 gene revealed the presence of numerous potential enhancer binding elements, including six NF-IL6-like sites, four AP-1, one AP-2, one NF-κB, two Sp1, two interferon-γ-activated sites (GAS), one Hox-1.3, and five binding sites for Ets family members. Transfection of BALB/c 3T3 cells with promoter DNA fragments linked to the luciferase reporter gene revealed that the 5′-flanking region of the TSG-14 gene comprises elements that can mediate a basal level of transcription and inducibility by TNF.

TNG-activated gene 14 (TSG-14)1 was originally isolated by differential hybridization of a cDNA library from TNF-treated normal human FS-4 foreskin fibroblasts (1). TSG-14 cDNA encodes a secreted glycoprotein of 42 kDa (2). An essentially identical cDNA termed PTX-3 was cloned by Brevisio et al. (3) from IL-1-stimulated human vascular endothelial cells. In its C-terminal half TSG-14 protein shows significant sequence homology (20–30%) with members of the pentraxin family of proteins (2, 3). The pentraxin proteins exhibit a characteristic discoid arrangement of five noncovalently bound subunits and share an eight-residue amino acid homology domain ("pentraxin signature"). The pentraxin family comprises two major acute phase proteins, the serum amyloid P component (SAP) and C-reactive protein (CRP) with a molecular mass of approximately 25 kDa (4, 5) and four larger proteins with molecular mass around 40–50 kDa: TSG-14/PTX3 (1–3, 6), Xenopus laevis protein (XL-PXN1) (7), an acrosomal protein (apexin) from guinea pig sperm (8), and a neuronal pentraxin protein (9). A remarkable feature of the smaller pentraxins is their high degree of conservation throughout evolution since they have been described in species as diverse as man, mouse, guinea pig, rat, rabbit, marine teleosts, and Limulus polyphemus, an invertebrate (10–13).

While apexin, XL-PXN1, and neuronal pentraxin have only recently been identified, SAP and CRP were described about 50 years ago and have been extensively studied. SAP and CRP are acute phase proteins whose serum concentrations increase dramatically in response to trauma or inflammation (4, 5). Human and murine CRP and SAP genomic DNAs have a similar organization and show significant sequence homology (14–17). However, CRP and SAP show some species specific differences in their regulation. CRP is highly inducible in humans but not in mice during inflammation, whereas SAP is the major acute phase protein in mice, but its concentration stays relatively constant in human serum (4). In order to elucidate the activation of these genes, their promoters have been analyzed structurally and functionally. Several studies have identified sequences in the 5′-flanking regions of the CRP and SAP genes that can mediate their inducibility by the inflammatory cytokines IL-1, TNF, and IL-6. Human and murine CRP genes were shown to contain in their 5′-flanking regions a TATA box along with binding sites for transcription factors of the C/EBP (NF-IL6) family that, along with the so-called acute phase response factor/Stat3, are thought to mediate cytokine inducibility (16, 18–23). The presence of specific binding sites for hepatocyte nuclear factor 1 in the CRP and SAP promoters was implicated in the hepatocyte-specific expression of these genes (19, 22).

The fact that IL-1 and TNF, two major mediators of inflammation, can induce transcriptional activation of the TSG-14 gene, as demonstrated by nuclear run-on analysis (2), and that TSG-14 is structurally related to two major acute phase proteins suggested that TSG-14 plays a role in the inflammatory response. Indeed, the appearance of TSG-14 protein in the serum was induced in mice after LPS injection (6). In addition, the concentration of TSG-14 was increased in the serum of...
human volunteers injected with LPS and in the sera of patients with bacterial septicemia (6). In both the human volunteers and in mice, immunoblot analysis of serum samples showed the top detectable TSG-14 protein before LPS stimulation, followed by the appearance of a prominent 42-kDa TSG-14 band, with peak levels detected at 4–6 h after injection with LPS. However, when attempts were made to identify the cellular source of TSG-14 in LPS-injected mice, no TSG-14 mRNA was detected in the liver and several other organs (spleen, kidney, and lung) (6). Alles et al. (24) recently demonstrated that TSG-14/PTX-3 is abundantly synthesized in cells of the human monocyte-macrophage lineage. Thus, TSG-14 is different from CRP, SAP, and other classical acute phase proteins produced by hepatocytes in that it is produced mainly by fibroblasts, endothelial cells, and monocytes.

Although human TSG-14/PTX-3 genomic DNA has been isolated and shown to contain three exons (3), no information on the promoter region was available. In order to investigate TSG-14 gene regulation and its tissue-specific expression, we decided to clone the murine TSG-14 genomic DNA and to analyze its 5' flanking region. In this study, we describe the cloning and sequencing of approximately 1.4 kb of the TSG-14 promoter, of the first exon, the first intron, the second exon, and part of the second intron. With the aid of a murine cell line that expresses TSG-14 after treatment with IL-1 or TNF, we determined the transcriptional start site of TSG-14 mRNA. Initial information obtained by transient transfection analysis of the TSG-14 promoter suggests a complex mechanism of regulation of gene expression. An interesting feature of the murine TSG-14 promoter is the presence of six potential NF-IL6 elements along with potential AP-1 and NF-kB binding sites. The absence of elements associated with the hepatocyte-specific expression of CRP and SAP is consistent with the lack of a significant level of TSG-14 gene expression in the mouse liver.

**EXPERIMENTAL PROCEDURES**

Screening of Genomic Library and Isolation of the Murine TSG-14 Genomic DNA—A genomic library from murine embryonic stem cells cloned into λGem 11 phage (kindly provided by Dr. Ulrike Müller, University of Zürich, Switzerland) was screened by hybridization with a radiolabeled EcoRI/DralI fragment (204 bp) from the 5' end of the human TSG-14 cDNA (2). Phage DNA from the positive plaques was purified and further characterized by restriction mapping and Southern blot analysis. A BamHI fragment of 2.7 kb that showed a strong hybridization with the 204-bp fragment from the 5' end of TSG-14 cDNA was selected and cloned into a pUC-derived plasmid, pGEM-2zf (+) (Promega Corp., Madison, WI). This fragment was partially sequenced on both strands with Sequenase (U.S. Biochemical Corp.). The rest was sequenced with the aid of the automated Applied Biosystem 373 DNA Sequencer at the Macromolecular Sequencing and Synthesis Laboratory, NYU Medical Center.

Cell Cultures and Analysis of mRNA Expression—BALB/c 3T3 murine fibroblasts (provided by Dr. Claudio Basilio, NYU Medical Center) were maintained in Dulbecco's modified Eagle's medium with 10% calf serum. Mouse embryonic fibroblasts isolated from 12-14-day-old embryos were grown in minimal essential medium with 10% fetal calf serum and used after two passages. Murine J 774 and J 774E cell lines of monocyte-macrophage origin were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The "63" murine monocyte-macrophage cell line was cultured in Dulbecco's modified Eagle's medium with 3% fetal calf serum and nonessential amino acids. The J 774, J 774E, and 63 cell lines were kindly provided by Dr. Stephanie Diment, NYU Medical Center. RNA was isolated from cultured cells as described before (2). Fifteen μg of RNA was denatured and blotted onto a nylon membrane (Nytran, Schleicher & Schuell) using a Hoefer PR 600 slot-blot apparatus (Hoefer Scientific Instruments, San Francisco, CA). Filters were hybridized with an Nco/HindIII fragment (486 bp; see Fig. 1A), containing the second exon of the murine TSG-14 gene, labeled with [α-32P]dCTP by nick translation. After the first hybridization, the filters were stripped and rehybridized with a labeled pH27 cDNA, encoding an invariant "housekeeping" mRNA (1). Filters were exposed to x-ray film at ~70 °C up to 7 days.

Transcriptional Start Site Mapping—For S1 nuclease analysis, total cytoplasmic RNA was isolated from BALB/c 3T3 cells treated for 4 h with 4 ng/ml recombinant murine TNF-α (kindly provided by Dr. Motomu of the Suntory Institute for Biomedical Research, Osaka, Japan) and from untreated control cells. Poly(A)+ mRNA was purified with the aid of the Oligotex-dT mRNA isolation kit (Qiagen, Chatsworth, CA). A 90-mer oligonucleotide spanning nucleotides −14 to +76 (relative to the subsequently determined transcription start site) was labeled at its 5' end with [γ-32P]ATP and T4 polynucleotide kinase (Promega). Digestion with S1 nuclease was performed for 30 min at 15 °C. After phenol-chloroform extraction and ethanol precipitation, the samples were run on a 6% sequencing gel along with labeled pGEM/Alul markers. For primer extension analysis, 2 μg of poly(A)+ mRNA from untreated or TNF-treated (50 ng/ml) BALB/c 3T3 cells, purified as described above, was hybridized overnight to an end-labeled 21-mer oligonucleotide spanning nucleotides +122 to +143. Extension was performed using 50 units of mouse mammary tumor virus reverse transcriptase, and further processing was done as described by Sambrook et al. (25). The samples were then analyzed on a 6% sequencing gel. Sequencing with the 21-mer oligonucleotide was performed for calibration.

Construction of the Different Plasmids—The BamHI fragment (containing 1438 bp of the flanking region of the TSG-14 gene) was inserted in front of the promoterless luciferase gene into the pGEM-2 vector kindly provided by Dr. Ed Ziff, NYU Medical Center. A series of 5'-unidirectional deletions of the promoter region was generated, taking advantage of unique restriction sites (see Fig. 1A). Briefly, the BamHI/Alul pGEM-2 construct was digested with Milul and with either SpeI or NdeI, purified, blunt-ended, and religated. The BglII construct was generated by limited digestion of the BamHI/Alul pGEM-2 construct, and the 6330-bp plasmid was purified and religated.

Transfection of BALB/c 3T3 Cell Line and Luciferase Assay—BALB/c 3T3 cells were seeded in 100-mm dishes (106 cells/dish) 18 h before transfection. Transfection was carried out by calcium phosphate precipitation with the aid of a transfection reagent (5 Prime, Inc., Boulder, CO). Two μg of each of the promoter constructs in the pGEM-2 plasmid and 100 ng of pCMV-β-encoding β-galactosidase under the control of the CMV promoter (Clontech, Palo Alto, CA) were used per plate. At 24 h after transfection, the medium was removed and replaced with fresh medium. Cells were allowed to recover for 24 h and were then stimulated with recombinant mouse TNF-α (100 ng/ml; provided by M. Tsujimoto of the Suntory Institute for Biomedical Research, Osaka, Japan) or with recombinant murine IL-1α (4 ng/ml, purchased from Biosource International, Camarillo, CA) for another 24 h. Cells were then harvested and lysed by three cycles of freezing and thawing in 100 μl Triton, pH 7.8, buffer. After centrifugation, the supernatant was recovered and used to determine the β-galactosidase activity. β-Galactosidase activity was measured by an enzymatic assay using O-nitrophenyl-β-D-galactopyranoside as a substrate. The absorbance was measured at 420 nm. Luciferase activity was determined with the aid of a Lumat LB9501 luminometer. Luciferase activity values were normalized to the β-galactosidase activity (relative luciferase activity). Statistical analysis was done by the test for paired samples.

**RESULTS**

Isolation of the Murine TSG-14 Genomic DNA—In order to isolate the mouse TSG-14 promoter region, we screened a mouse genomic library with a 204-bp probe (EcoRI/DralI fragment) comprising the 5' end of the human TSG-14 cDNA. After four rounds of plaque screening, one positive plaque was isolated from a total of approximately 5 × 109 plaques screened, and the DNA from the phage was purified. Southern blot and restriction enzyme analysis of the phage DNA showed that a 2.7-kb BamHI/BamHI fragment cross-hybridized strongly with the 204-bp human TSG-14 cDNA probe but not with a cDNA probe that contains 330 bp from the third exon, while another genomic fragment of approximately 5 kb cross-hybridized with the human cDNA probe from the third exon but not with the 204-bp probe (data not shown). These results suggested that the 2.7-kb fragment contains at least part of the promoter region of TSG-14 and that the 5-kb fragment contains the third

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2 G. W. Lee, A. F. Suffredini, and J. Višček, unpublished data.
exon (or part of the third exon) of the mouse TSG-14 gene. Since our main aim was to isolate the promoter region of the TSG-14 gene, we proceeded to subclone the 2.7-kb fragment and to determine its sequence. Comparison of the nucleotide sequence of the 2.7-kb fragment with the sequence of the human TSG-14 cDNA (2), together with information on the genomic organization of the human TSG-14 gene (3), allowed us to determine the genomic organization of the mouse gene. The 2.7-kb fragment comprised 1374 bp of the 5′-flanking region, the first exon (265 bp) followed by an intron of 447 bp, the second exon (402 bp), and part of the second intron (221 bp) (Fig. 1, A and B). Interestingly, the first and second exons of mouse TSG-14 show an overall 80% identity at the nucleic acid level with the first and second exons of human TSG-14. At the protein level, there is 88% identity (93% homology) between predicted sequences encoded by murine and human exon 1 and 75% identity (81% homology) between human and murine exon 2 sequences (Fig. 2). This high degree of homology suggests that TSG-14 is well conserved during evolution, as is also true for other members of the pentraxin family of proteins (10–13).

In the promoter region of TSG-14, we were unable to find consensus sequences for either a TATA-box or a CCAAT-box. However, we have determined the presence of several consensus binding sequences for known transcription factors. Among these sequences, we have identified six NF-IL6-like recognition sequences, four AP-1, one AP-2, one NF-κB, two Sp1, two GAS, one Hox-1.3 site, and five consensus sequences for the binding of different members of the Ets family of transcription factors (Fig. 1B, Table I). Another intriguing element found in the TSG-14 promoter is an alternating CA residues stretch of 44 located between nucleotides 2645 and 2601. A similar sequence has been found in several other genes such as the 5′-flanking region of the iNOS gene (26) and the Scya5 gene (27). It has been suggested that such “TG element” sequence may potentially have a Z-DNA conformation and serve as an enhancer element (28).

Selection of Murine Cell Lines that Express TSG-14 mRNA after Induction by TNF and IL-1—In order to determine the transcription start site and to analyze the promoter activity of the 5′-flanking region of TSG-14, we first sought to identify murine cells that express endogenous TSG-14 mRNA after treatment with IL-1 or TNF. Since previous results demonstrated that TSG-14 is expressed mostly in fibroblasts, endothelial cells, and cells of the monocyte-macrophage lineage (2, 24), we investigated the induction of TSG-14 mRNA in cultured murine embryo fibroblasts and in a panel of different murine cell lines of fibroblast and monocyte-macrophage origin (Fig. 3, Table II). Among all the cell lines tested, TSG-14 was inducible only in mouse embryo fibroblasts, as already described (2), and in BALB/c 3T3 fibroblasts. The lack of inducible TSG-14 mRNA expression in most permanent cell lines is similar to the earlier demonstrated absence of TSG-14 inducibility in a majority of transformed human cell lines examined (2). We therefore decided to use BALB/c 3T3 cells for our further experiments.

Determination of the Transcriptional Start Site—Mapping of the transcriptional start site was done by S1 nuclease protection assay. Poly(A)+ mRNA was isolated from control and TNF-treated BALB/c 3T3 cells as described under “Experimental Procedures” and hybridized with a 90-mer oligonucleotide spanning nucleotides −14 to +76 (the numbering is relative to
Fibroblasts; lanes 3 and 7 then stripped and rehybridized with a probe for Hc7 cDNA hybridizing containing the second exon of the murine TSG-14 gene (TSG-14) and durers. The blot was first hybridized with a

Numbers in parentheses indicate number of residues matching consensus sequence/total number of residues.

A

Fig. 3. Analysis of TSG-14 mRNA expression in different murine cell lines. Cells were treated for 4 h with TNF (50 ng/ml) or IL-1 (2 ng/ml) or left untreated. RNA was isolated, separated, and immobilized on nylon membranes as described under “Experimental Procedures.” The blot was first hybridized with a NcoI/HindIII fragment containing the second exon of the murine TSG-14 gene (TSG-14) and then stripped and rehybridized with a probe for pH7 cDNA hybridizing with a housekeeping mRNA (pH7). A, lanes 1 and 2, mouse embryonic fibroblasts; lanes 3 and 4, BALB/c 3T3 cells; lanes 5 and 6, J 774E cells; lanes 7 and 8, 63 cells; lanes 9 and 10, J 744E cells; lanes 1, 3, 5, 7, and 9, untreated cells; lanes 2, 4, 6, 8, and 10, TNF-treated cells. B, lane 1, mouse embryonic fibroblasts; lane 2, BALB/c 3T3 cells; lane 3, J 774 cells; lane 4, 63 cells; lane 5, J 744E cells. All samples in panel B are from IL-1-treated cells.

the subsequently determined transcriptional start site). A band of approximately 70 nucleotides was protected from digestion by S1 nuclease when poly(A) + mRNA from cells treated with TNF was annealed to the 90-mer oligonucleotide, but not when poly(A) + mRNA from untreated cells was used (Fig. 4A). These findings suggest that there is one major transcription start site located about 70 bp upstream of the nucleotide +76. In order to confirm this result and to determine the precise nucleotide at which transcription starts, we performed primer extension analysis using a 21-mer oligonucleotide corresponding to nucleotides +122 to +143. One major transcription start site was revealed, located 135 bp upstream of the translation start site, which is in agreement with the results of S1 nuclease mapping (Fig. 4B). This nucleotide, designated +1 in Fig. 1, corresponds to an adenine residue. The sequence surrounding nucleotide +1 matches 5 out of 7 nucleotides with the loose consensus sequence defining an initiator element (PyPyA +1 ) matched 5 out of 7 nucleotides with the loose consensus sequence defining an initiator element (PyPyA +1 ). Therefore, the presence of this sequence in the 5′-flanking region of TSG-14 would confer inducibility by TNF in BALB/c 3T3 cells, the

| Treatment | Sequence in TSG-14 | Position |
|-----------|-------------------|----------|
| AP-1      | TGA(G/C)T(C/A)A   | −1099    |
| AP-2      | CCC(A/C)N(G/C)(G/C)(G/C) | −865    |
| GAS       | TTCN (G/C)T(C/A)A | −1099    |
| NF-IL6    | T(G/T)NNGNAT      | −122     |
| NF-κB     | GGG(A/G)NN(C/T)(C/T)(C/T)CC | −122     |
| Sp1       | (G/T)(G/A)G(C/A)G(T)/G(A)(G/A)(C/T) | −122     |
| ETS FAMILY | AGGAA (G/A) | −975     |
| PEA3      | AGGAAA (G/A)     | −975     |
| Ets-1     | (A/G)C(C/A)GGA(T/A)(G/A)(T/C)TCAGGATGT | −975     |
| Pu. 1     | GAGGAA (G/A)     | −975     |
| HOX-1.3   | C(C/T)(C/T)NATTA(T/G)(T/C)ACTAATGTAA | −975     |

a Consensus binding sequences are based on published data, as follows: AP-1 (45), AP-2 (46), GAS (39), NF-IL6 (47), NF-κB (48), Sp1 (49), PEA3 (50), Ets-1 (51), Pu.1 (52), HOX-1.3 (53).

b Numbers in parentheses indicate number of residues matching consensus sequence/total number of residues.

| Treatment | Sequence in TSG-14 | Position |
|-----------|-------------------|----------|
| AP-1      | TGA(G/C)T(C/A)A   | −1099    |
| AP-2      | CCC(A/C)N(G/C)(G/C)(G/C) | −865    |
| GAS       | TTCN (G/C)T(C/A)A | −1099    |
| NF-IL6    | T(G/T)NNGNAT      | −122     |
| NF-κB     | GGG(A/G)NN(C/T)(C/T)(C/T)CC | −122     |
| Sp1       | (G/T)(G/A)G(C/A)G(T)/G(A)(G/A)(C/T) | −122     |
| ETS FAMILY | AGGAA (G/A) | −975     |
| PEA3      | AGGAAA (G/A)     | −975     |
| Ets-1     | (A/G)C(C/A)GGA(T/A)(G/A)(T/C)TCAGGATGT | −975     |
| Pu. 1     | GAGGAA (G/A)     | −975     |
| HOX-1.3   | C(C/T)(C/T)NATTA(T/G)(T/C)ACTAATGTAA | −975     |

a Consensus binding sequences are based on published data, as follows: AP-1 (45), AP-2 (46), GAS (39), NF-IL6 (47), NF-κB (48), Sp1 (49), PEA3 (50), Ets-1 (51), Pu.1 (52), HOX-1.3 (53).

b Numbers in parentheses indicate number of residues matching consensus sequence/total number of residues.

| Treatment | Sequence in TSG-14 | Position |
|-----------|-------------------|----------|
| AP-1      | TGA(G/C)T(C/A)A   | −1099    |
| AP-2      | CCC(A/C)N(G/C)(G/C)(G/C) | −865    |
| GAS       | TTCN (G/C)T(C/A)A | −1099    |
| NF-IL6    | T(G/T)NNGNAT      | −122     |
| NF-κB     | GGG(A/G)NN(C/T)(C/T)(C/T)CC | −122     |
| Sp1       | (G/T)(G/A)G(C/A)G(T)/G(A)(G/A)(C/T) | −122     |
| ETS FAMILY | AGGAA (G/A) | −975     |
| PEA3      | AGGAAA (G/A)     | −975     |
| Ets-1     | (A/G)C(C/A)GGA(T/A)(G/A)(T/C)TCAGGATGT | −975     |
| Pu. 1     | GAGGAA (G/A)     | −975     |
| HOX-1.3   | C(C/T)(C/T)NATTA(T/G)(T/C)ACTAATGTAA | −975     |

a Consensus binding sequences are based on published data, as follows: AP-1 (45), AP-2 (46), GAS (39), NF-IL6 (47), NF-κB (48), Sp1 (49), PEA3 (50), Ets-1 (51), Pu.1 (52), HOX-1.3 (53).

b Numbers in parentheses indicate number of residues matching consensus sequence/total number of residues.
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Fig. 5. Transcriptional activity of the TSG-14 promoter and its deletion constructs linked to the luciferase reporter gene upon transfection into BALB/c 3T3 cells. A, induction into BALB/c 3T3 cells. A, the deletion constructs shown were used for transfection of BALB/c 3T3 cells. After transfection cells were either left untreated or treated with TNF (100 ng/ml) for 24 h. The relative luciferase activity was determined by normalizing to the β-galactosidase activity. Fold induction represents the ratio of relative luciferase activity in TNF-treated over control cells. Each value is the mean and standard deviation from six independent experiments. p values were determined by the t test for paired samples. B, actual results representative of the experiments summarized in panel A. Means and standard deviations of three independent experiments are shown. Base-line relative luciferase activity (i.e. value obtained after transfection of cells with “empty” luciferase vector) was approximately 3000 and did not increase after TNF treatment.

−1374 to +84) was cloned in front of the promoterless luciferase reporter gene. A series of 5′-unidirectional deletions were also generated to determine which region(s) of the promoter are important for TSG-14 induction (Fig. 5A). These different constructs were transfected into BALB/c 3T3 cells and tested for their response to TNF. Relative transcriptional activity was determined by normalizing luciferase to β-galactosidase activity. All constructs mediated a significant basal level of transcription (background relative luciferase activity with the empty vector was approximately 3000), in agreement with the basal level of transcription observed in untreated cells by primer extension analysis (Fig. 4B). The mean induction by TNF of the BamHI and the BspMI constructs was 2.2- and 2.7-fold, respectively. Although this induction is low, it was reproducible and statistically significant (Fig. 5A). Removing more of the 5′-flanking region down to −436 or −213 decreased inducibility by TNF, suggesting the presence of positive regulatory elements between positions −436 and −1374. The decrease in inducibility by TNF with decreasing lengths of the promoter constructs was accompanied by a gradual increase in the basal level of transcription (Fig. 5B), suggesting a complex pattern of regulation involving both positive and negative regulatory elements.

FIG. 4. Determination of the transcription start site of the TSG-14 gene by S1 nuclease mapping and primer extension. A, S1 nuclease mapping. Poly(A)+ mRNA isolated from untreated BALB/c 3T3 cells (CTRL) or from cells treated with TNF for 4 h (TNF) was annealed to a 90-mer oligonucleotide spanning nucleotides −14 to +76 of the TSG-14 gene. The lane marked PROBE was loaded with the labeled 90-mer oligonucleotide alone. The pGEM plasmid digested by AluI was used in the lane labeled MARKERS. The arrow points to the band obtained by S1 mapping. B, primer extension. Poly(A)+ mRNA from control (CTRL) or TNF-treated BALB/c 3T3 cells (TNF) was hybridized to a 21-mer oligonucleotide corresponding to positions +122 to +143. Sequencing with this oligonucleotide was used for calibration. The start site of transcription is marked by an arrow, and the corresponding nucleotide is indicated by an asterisk.
Human TSG-14 cDNA encodes a 42-kDa glycoprotein with significant sequence homology to the acute proteins CRP and SAP and other members of the pentraxin family (2). The fact that TSG-14 is inducible by the proinflammatory cytokines TNF and IL-1, together with the demonstration that TSG-14 protein levels are elevated in the serum of mice and humans after LPS injection (6), strongly suggests that TSG-14 plays a role in inflammation. To date no information regarding TSG-14 gene regulation by TNF and IL-1 has been available. Therefore, we cloned and sequenced a 2.7-kb fragment of the murine genomic TSG-14 DNA including −1.4 kb of its 5′-flanking region. A comparison of the intron-exon structure of the isolated portion of the murine gene with the earlier published organization of the human TSG-14/PTX-3 gene (3) revealed a close similarity. In addition, a high degree of homology was found between the coding sequences of the murine DNA and the earlier determined human TSG-14 cDNA sequence (1–3).

At the predicted amino acid level there is 88% identity (93% homology, if conservative substitutions are included) and 75% identity (81% homology) between sequences encoded by murine and human exons 1 and 2, respectively. Hence, as is also true for other pentraxins (10–13), TSG-14 is highly conserved during evolution, suggesting structural constraints imposed by protein function.

Analysis of the 5′-flanking sequence of the murine TSG-14 gene revealed no obvious consensus sequence for a TATA- or a CAAT-box. However a sequence that matches five out of seven residues of the consensus sequence for initiator elements (29, 30) defines the transcriptional start site in the TSG-14 promoter. Furthermore, sequence analysis revealed the presence of several potential cis-acting elements in the 5′-flanking region of the TSG-14 gene, including six NF-IL6-like sequences, four AP-1 sites, one AP-2, one NF-κB, two Sp1, and two GAS (Fig. 1B, Table I). Studies on the regulation of other TNF- or IL-1-inducible genes revealed that NF-κB, NF-IL6 (C/EBP-family), and AP-1 transcription factors (and cooperative interactions among these factors) are often crucial in their regulation (31–35). The presence of multiple potential NF-IL6 binding sites in the TSG-14 promoter is of particular interest in view of the central role played by NF-IL6 in the acute phase response and inflammation (36–38). Whether the GAS elements are likely to be functional is uncertain because the human TSG-14 gene is not inducible by interferon-γ, nor did interferons affect TSG-14 gene expression induced by other cytokines (1). However, variants of the GAS element serve as binding sites for various Stat family proteins, important in the signaling from a variety of cytokine and growth factor receptors (39). Another potential binding site identified in the murine TSG-14 promoter is the Hox-1.3 consensus element. The homebox gene-encoded Hox-1.3 protein is expressed mainly during embryogenesis but also in several adult tissues, and its expression in fibroblasts seems to correlate with cell growth (40). Several consensus binding sequences for transcription factors of the Ets family have also been identified in the TSG-14 promoter. Similar sequences have been found in a number of cytokine genes, including the TNF-α promoter in which it is thought to play a crucial role (41). Similarly, the binding of the Pu.1 transcription factor in the IL-1 promoter appears to be important for the cell-specific expression of IL-1 (42).

Another interesting element in the murine TSG-14 promoter is the 44-bp stretch of alternating CA residues. Enhancing functions have been attributed to this element (28). Intriguingly enough, the same type of sequence has been found in the intron of the human, but not the mouse, CRP gene, and it has been hypothesized that this site may be involved in the differential expression of CRP in human and mouse cells (43). While human and mouse CRP and SAP genes contain in their promoter region at least one consensus sequence for the liver-specific transcription factor hepatocyte nuclear factor 1, no such sequence was found in the TSG-14 promoter. This result is in agreement with the finding that, in contrast to CRP and SAP, TSG-14 is not produced by the liver (6, 24).

Initial analysis of promoter function of the TSG-14 gene by transfection into BALB/c 3T3 cells of the 5′-flanking region linked to the luciferase gene showed that the −1.4-kb DNA fragment mediates a relatively high level of constitutive transcriptional activity along with some inducibility by TNF. Deletion analysis suggests that removal of sequences upstream of position −436 resulted in a somewhat diminished inducibility by TNF (Fig. 5B). Even more pronounced than the decrease in inducibility by TNF was a concurrent increase in constitutive transcriptional activity, suggesting the presence of upstream negative regulatory sequences. Analysis of other promoters showed that NF-IL6 sites can function both as positive and negative regulatory elements (44). It is conceivable that some of the multiple potential NF-IL6 binding sites in the murine TSG-14 promoter will also turn out to act as negative elements. Additional investigations by deletion analysis and site-directed mutagenesis will be required to define more precisely the cis-acting elements and trans-acting factors important in the induced expression of the murine TSG-14 gene.

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