A High Affinity HSF-1 Binding Site in the 5′-Untranslated Region of the Murine Tumor Necrosis Factor-α Gene Is a Transcriptional Repressor*

Tumor necrosis factor-α (TNFα) is a pivotal early mediator of host defenses that is essential for survival in infections. We previously reported that exposing macrophages to febrile range temperatures (FRT) (38.5–40 °C) markedly attenuates TNFα expression by causing abrupt and premature cessation of transcription. We showed that this inhibitory effect of FRT is mediated by an alternatively activated repressor form of heat shock factor 1 (HSF-1) and that a fragment of the TNFα gene comprising a minimal 85-nucleotide (nt) proximal promoter and the 138-nt 5′-untranslated region (UTR) was sufficient for mediating this effect. In the present study we have used an electrophoretic mobility shift assay (EMSA) to identify a high affinity binding site for HSF-1 in the 5′-UTR of the TNFα gene and have used a chromosome immunoprecipitation assay to show that HSF-1 binds to this region of the endogenous TNFα gene. Mutational inactivation of this site blocks the inhibitory effect of overexpressed HSF-1 on activity of the minimal TNFα promoter (−85/+138) in Raw 264.7 murine macrophages, identifying this site as an HSF-1-dependent repressor. However, the same mutation fails to block repression of a full-length (−1080/+138) TNFα promoter construct by HSF-1 overexpression, and HSF-1 binds to upstream sequences in the regions −1080/−845, −533/−196, and −326/−39 nt in EMSA, suggesting that additional HSF-1-dependent repressor elements are present upstream of the minimal −85-nt promoter. Furthermore, although mutation of the HSF-1 binding site in the minimal TNFα promoter construct abrogates HSF-1-mediated repression, the same mutation fails to abrogate repression of this construct by high levels of HSF-1 overexpression or exposure to 39.5 °C. This suggests that HSF-1 might repress TNFα transcription through redundant mechanisms, some of which might not require high affinity binding of HSF-1.

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† The abbreviations used are: TNF, tumor necrosis factor α; LPS, bacterial endotoxin lipopolysaccharide; HSF, heat shock protein; HSF, heat shock factor; rHSF, recombinant HSF; HRE, heat shock response element; UTR, untranslated region; IL, interleukin; EMSA, electrophoretic mobility shift assay; FRT, febrile range temperature; nt, nucleotide(s); NF, nuclear factor; ChIP, chromosomal immunoprecipitation; STAT, signal transducers and activators of transcription.

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ing sequence. It does, however, contain multiple nGAAn elements positioned in critical locations, including adjacent to an essential Sp-1 binding site, at the transcription start site, and 35 nucleotides downstream of the transcription start site. In the present study we have identified the high affinity binding site for HSF-1 in the minimal temperature-responsive TNFα gene sequence and showed that inactivating the site by mutation reverses the repression of this promoter fragment by HSF-1 overexpression.

**EXPERIMENTAL PROCEDURES**

**Primers, Oligonucleotides, and Probes—** All oligonucleotides were synthesized by Invitrogen, Gaithersburg, MD. Fig. 1 (see below) shows the sequences of each oligonucleotide used for electrophoretic mobility shift assays (EMSA). Complementary oligonucleotides were synthesized, annealed, and used as probe for EMSA. Primers for PCR-directed mutagenesis were Luc_5, 5'-gggagagtagtggctttcgtc-3' (5'-pGL3 backbone); Luc_3, 5'-ctagcaaaataggctgtccc-3' (luciferase open reading frame); 49_Mut forward primer, 5'-gggagagtagtggctttcgtc-3' and 49_Mut reverse primer, 5'-gggagagtagtggctttcgtc-3'. For EMSA, full-length double-stranded TNFα gene sequence was isolated from frozen stocks. All media and reagents contained 3.5% bovine serum albumin (Bio-Rad, Hercules, CA) with bovine serum albumin as standard. Double-stranded oligonucleotides were radiolabeled using T4 polynucleotide kinase (Promega) and [γ-32P]ATP according to the manufacturer's protocol. EMSA reactions containing 5 μg of nuclear extract or the indicated amount of recombinant TNFα-1, 0.65 pmol of radiolabeled oligonucleotide, 1 μg of poly(dI/dC), 10 μM Tris-HCl (pH 7.8), 10% glycerol, 5 mM EDTA, and 1 mM diithiothreitol in volume of 20 μl were incubated at room temperature for 30 min. Where indicated, excess unlabeled competitor double-stranded oligonucleotide or 1 μl of anti-HSF-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with the nuclear extracts for 30 min at room temperature before the addition of the radiolabeled probe. The DNA-protein complexes were then electrophoretically resolved on 4% nondenaturing polyacrylamide gels. The dried gels were analyzed by phosphorimaging (PhosphorImager, Molecular Dynamics) and subsequently exposed to x-ray film.

**Chromosomal Immunoprecipitation Assay—** ChIP assay was performed using a kit from Upstate Biotechnology Inc. (Lake Placid, NY). Unless otherwise stated, all reagents were provided in the kit. In brief, Raw 264.7 cells were infected with 0.1 M NaHCO3 and 1% SDS. Protein-DNA cross-links were reverted by incubating at 65 °C for 4 h, and after proteinase K digestion, DNA was extracted with phenol-chloroform and precipitated using ethanol. PCR was performed (30 cycles of 30 s at 94 °C, 1 min at 50 °C, and 1 min at 72 °C for 45 s) using primers specific for the murine TNFα sequence between –85 and +138: 5'-ggatcctgtgctagcttccggagggtt-3' (forward) and 5'-gaagtga (reverse) (see Fig. 4). PCR products were gel-purified and cloned into the pCRII vector (Invitrogen, Gaithersburg, MD) and expressed in E. coli. The fusion protein was cleaved using thrombin, and the purified His-tagged protein was used for the EMSA as a positive control, immunoprecipitated DNA was also amplified using PCR primers specific for the HNF-3β-containing 190 nt fragment of the murine HNF-3β promoter 5'-agaatgaga (forward) and 5'-gaagtga (reverse) using the same PCR conditions as for TNFα except for a 60 °C annealing temperature.

**Transfection and Reporter Gene Analysis—** Cells were transfected using FuGENE 6 (Roche Molecular Biochemicals). 4 μg of each test plasmid and 0.5 μg of control (pRL-SV40, Promega) plasmid DNA were mixed with 15 μl of FuGENE 6 in 100 μl of medium. The mixture was incubated at room temperature for 15 min and then added to cells in 60-mm dishes. After 24 h, the cells were split into 24-well plates (1:12 per 60-mm plate). After an additional 24 h, the cells were stimulated with LPS at 37 °C or 39.5 °C for 6 h. Cells were lysed, and reporter gene expression was analyzed using the Dual Luciferase Reporter assay kit (Promega) according to the manufacturer's protocol.

**Statistical Analysis—** Data are presented as means ± S.E. Differences between two groups of data were analyzed using the unpaired Student t test. Differences among more than two groups were tested by applying the Fisher protected least significant differences test applied to a one-way analysis of variance.

**RESULTS**

**Organization of the Murine Minimal Promoter and 5'-Untranslated Region—** We previously reported that the murine TNFα gene sequence spanning –85 to +138 nt bound HSF-1 in EMSA competition assays and, when transfected into Raw 264.7 macrophages, conferred transcriptional repression by febrile range temperature (FRT; 39.5 °C) or HSF-1 overexpression (15). The high affinity binding sequence for HSF-1 com-
High Affinity Binding of HSF-1 to the TNFα 5′-UTR

A

HRE consensus nGAAnnTTCn

B

HRE sequence from the human HSP70 promoter (24)

-105 GATCTCGGCTGGATAATCCCACCTTGGCACGCGA

C

Mouse TNFα Minimal Promoter/5′UTR Sequence:

\[
\begin{array}{c}
\text{Sp1} \\
\text{TATA}
\end{array}
\]

-85 CGAGGGTGA A7GAGGCT_TCTCCTCACC TTATCAA GGGCTAATAA -83/43

-35 AGGGCGGTG TCAGACACCC AGGCAGCAGA AGCTCCTCTCA GCAGGGACAG -15/5

16 CAAGGGACTA GCCAGAGGA GAAAGAAAC TCCAGACAT_CCTGGAAATA 30/68

66 GTCTCAGAA AAGCAAGCAG CCAACAGGCC AGGTTCTGTC_CCTTCACTC 65/85

101/121

D

Comparison of Mouse and Human TNFα +30/+68 Sequence:

+30 GCAGGGAGAAGACACGACTCCAGAACATCTTGAAA Mouse TNFα

+30 AGAGGGAGAAGGACACTGACAGACCACCCTTCGAAA Human TNFα

prizes a minimum of two nGAAn elements arranged as an inverted dyad repeat (20) (Fig. 1A). Fig. 1 shows the location of nGAAn elements in the –85 to +138 nt region of the murine TNFα promoter (Fig. 1C), the sequence of the heat shock response element (HRE) from the human HSP70 promoter (24) (Fig. 1B) and the sequences of each of the TNFα oligonucleotides used for the EMSA analysis of HSF-1 binding (Fig. 1C, underlined sequences). This fragment of the TNFα gene contains 10 nGAAn elements, but none arranged with perfect inverted dyad symmetry. Single nGAAn elements are positioned next to the Sp-1 binding site (–65 nt), at the transcription start site (–5 nt), and at nt +72 and +108 in the 5′-UTR. The 5′-UTR sequence spanning +30 to +68 contains an array of four nGAAn elements, three of which would form a perfect HRE if the “CT” sequence at nt +57 and +58 were inverted to “TC.” In our EMSA analysis, we probed with oligonucleotides spanning –83–43 and containing three nGAAn elements and the Sp-1 binding sequence, –15/+5 containing one nGAAn and the transcription start site, +30/+68 and containing the imperfect HRE, and +65/+85 and +101/+121, each containing one nGAAn element.

Identification of the High Affinity HSF-1 Binding Sites in the Murine TNFα Gene—The capacity of each nGAAn-containing sequence in the minimal TNFα promoter/5′-UTR to bind HSF-1 was analyzed by EMSA using the oligonucleotides listed in Fig. 1 using the following three-step strategy. First, the capacity of a 100-fold excess of each oligonucleotide to compete and block HSF binding to a consensus HSF binding sequence was analyzed using the sequence from the human HSP70 promoter as a radiolabeled probe and nuclear extracts from Raw 264.7 macrophages exposed to 39.5 °C for 1 h as a source of HSF-1, we found that unlabelled wild-type +30/+68 added at a 10-fold molar excess was sufficient to abrogate binding to radiolabeled +30/+68 (lane 2). Mutating the nGAAn sequence at +59 (59_Mut, lanes 9–12) did not change the capacity of +30/+68 to compete for HSF-1 binding. In striking contrast, 49_Mut failed to compete for HSF-1 binding. To confirm that HSF-1 binds with high affinity to the +30/+68 TNFα sequence, we repeated the EMSA analysis using each TNFα oligonucleotide as a radiolabeled EMSA probe (Fig. 2B). Of the TNFα sequences studied, only +30/+68 bound HSF-1 (lane 4), forming a complex that comigrated with the complex that formed on the HRE sequence from the HSF-1 promoter (lane 1). Shiftig with anti-HSF-1 antibody (lane 7) confirmed that the observed complex contained HSF-1. To further confirm that HSF-1 could directly bind to TNFα +30/+68, we repeated the EMSA analysis with purified recombinant human HSF-1 (Fig. 3). TNFα +30/+68 bound rHSF-1 (lanes 7–10), but to a lesser degree than the HSP70 HRE (lanes 2–5). By comparison, the other TNFα sequence oligonucleotides failed to detectably bind rHSF-1 (data not shown).

Mutational Inactivation of the HSF-1 Binding Site in TNFα 30/68—The +30/+68 TNFα sequence contains four nGAAn sites that form two possible partially overlapping HREs centered on nt +49 and +59, respectively (Fig. 1C). To further define the binding site for HSF-1 in this sequence we replaced the GAA sequences at either 50–52 nt (49_Mut) or 60–62 nt (59_Mut) with CCC (Fig. 4A) and analyzed the ability of the mutated +30/+68 oligonucleotides to bind HSF-1 in an EMSA competition assay (Fig. 4B). Using the wild-type +30/+68 oligonucleotide as the radiolabeled probe and nuclear extracts from Raw 264.7 cells exposed to 39.5 °C for 1 h as a source of HSF-1, we found that unlabelled wild-type +30/+68 added at a 10-fold molar excess was sufficient to abrogate binding to radiolabeled +30/+68 (lane 2). Mutating the nGAAn sequence at +59 (59_Mut, lanes 9–12) did not change the capacity of +30/+68 to compete for HSF-1 binding. In striking contrast, 49_Mut failed to compete for HSF-1 binding when added at 10- to 100-fold excess (lanes 5–7) and only partially competed when added at 1000-fold excess (lane 8). Direct binding of HSF-1 to radiolabeled probe containing the wild-type and mutated +30/+68 sequences (Fig. 3C) confirmed the results of the EMSA competition analysis. Although 59_Mut formed a complex (lane
High Affinity Binding of HSF-1 to the TNFα 5′-UTR

Effect of Mutational Inactivation of HSF-1 Binding on TNFα Transcriptional Activity—The functional significance of abrogating high affinity HSF-1 binding to the TNFα 5′-UTR in comparison with transcriptional repression was analyzed by introducing the GAA to CCC mutation at nt 50–52 into each of three TNFα promoter-driven luciferase reporter constructs: 1) the minimal promoter/5′-UTR (pTNF50–52 (Fig. 7A); 2) a 382-nt promoter/5′-UTR fragment (pTNF244–52) containing the most proximal NF-κB response element (Fig. 7B); and 3) a full-length 1.2-kb promoter/5′-UTR fragment (pTNF1080–(52) (Fig. 7C)). Each of these promoter fragments was cloned into the NheI/HindIII site of pGL3, transiently transfected into Raw 264.7 cells, and the promoter activity was compared in 37° and 39.5 °C cell cultures and in the presence of increasing concentrations of an HSF-1 expression plasmid at 37 °C (15). The analysis was performed in both the presence and absence of LPS. Although TNFα promoter activity was higher in the presence of LPS, the pattern of FRT- and HSF-1-induced effects on wild-type and mutated TNFα reporter constructs was comparable in LPS-stimulated and unstimulated cells. The data from the LPS-stimulated cells are shown. HSF-1 overexpression reduced the activity of both wild-type pTNF1080–52 (Fig. 7C) and pTNF50–52 (Fig. 7A), as we have previously reported (15), and similarly reduced the activity of pTNF244–52 (Fig. 7B). Mutation of the high affinity HSF-1 binding site (at nt 50–52) in pTNF50–52 seemed to interfere with the inhibitory effect of HSF-1 overexpression (Fig. 7A). At 1 and 2 μg of HSF-1 expression plasmid, the activity of the wild-type pTNF50–52 construct was decreased by 28 and 40%, respectively, whereas, activity of the mutant construct was unchanged. However, cotransfection with 3 μg of HSF-1 plasmid or incubation at 39.5 °C inhibited the activity of the wild-type and mutated pTNF50–52 construct by a similar extent.

HSF-1 overexpression inhibited both wild-type and mutated pTNF244–52 (Fig. 7B), but the extent of inhibition of the mutated construct was significantly less than that of the wild-type construct at 2 μg (22 versus 42%; p < 0.05) and 3 μg of HSF-1 plasmid (36 versus 59%; p < 0.05). Both constructs were inhibited to a similar extent by exposing cells to 39.5 °C. In contrast, in the full-length promoter (pTNF1080–52) (Fig. 7C), mutation of the high affinity HSF-1 binding site in the 5′-UTR had no detectable effect on the inhibitory effects of HSF-1 overexpression or exposure to 39.5 °C.

Interaction of HSF-1 with TNFα Promoter Sequences Upstream of −85—The transfection results suggested that additional HSF-1-responsive repressor elements might be present in the TNFα sequence upstream of the minimal promoter. To determine if HSF-1 binds to sequences upstream of −85, we prepared five partially overlapping fragments by PCR amplification spanning the region −39 to −1080. The amplified fragments were radiolabeled and used as a probe in EMSA using 39.5 °C for 60 min. DNA was sonicated to yield fragments of ~500-nt length. PCR with primers specific for the HRE-containing murine HSP70 promoter sequence amplified detectable product of the predicted 180-nt length in samples immunoprecipitated with each of the anti-HSF-1 antibodies (Fig. 6, lanes 3, 4), whereas no PCR product was detectable in samples immunoprecipitated without antibody (lane 5) or with an irrelevant rabbit anti-IL-13 antibody (lane 2), thereby validating the ChIP assay in this model. PCR amplification using primers specific for the TNFα sequence spanning −85 to +138, which includes the putative HSF-1 binding site, generated a detectable product of the predicted 223-nt length in samples immunoprecipitated with the anti-HSF-1 antibodies (Fig. 6, lanes 8, 9) but not in samples immunoprecipitated without antibody (lane 10) or with anti-IL-13 antibody (lane 7).

Binding of HSF-1 to the Endogenous TNFα Gene—Although we have demonstrated that HSF-1 binds to naked DNA under artificial cell-free conditions, its ability to repress TNFα transcription in the cell requires it to bind to the endogenous TNFα gene in vivo. We used the ChIP assay to determine if HSF-1 binds to the TNFα gene in vivo in Raw 264.7 cells incubated at...
purified rHSF-1 (Fig. 8). The PCR fragments $-1080/-845$ (235 bp, lane 1), $-533/-196$ (337 bp, lane 4), and $-326/-39$ (287 bp, lane 5) formed complexes with rHSF-1, whereas fragments $-889/-562$ (237 bp, lane 2) and $-686/-494$ (192 bp, lane 3) failed to form any detectable complex with rHSF-1.

**FIG. 4.** EMSA analysis of HSF binding to wild-type and mutated TNFα oligonucleotide +30/+68. A, the sequences of the wild-type and two mutated +30/+68 oligonucleotides. B, competition for binding to wild-type +30/+68. Nuclear extracts from Raw 264.7 cells exposed to 39.5°C for 1 h were used as a source of HSF-1 and probed with radiolabeled wild-type +30/+68. Each unlabeled oligonucleotide was added at the indicated molar excess. The HSF-DNA doublet band is indicted by the arrow. C, direct binding of HSF to radiolabeled wild-type and mutated TNFα oligonucleotides. The complex on +30/+68 was supershifted with anti-HSF-1 antibody (lane 2), and the supershifted complex is represented by the arrowhead.

**DISCUSSION**

In our earlier studies, we showed that TNFα expression is reduced in human and murine macrophages upon exposure to febrile temperature (10, 12, 15, 16) and that the effect is largely caused by a reduction in TNFα transcription in the warmer
HSF-1 binds to the murine with HSF-1-sufficient littermates. We previously showed that the HRE-containing Immnoprecipitates were analyzed by PCR using primers specific for approximately 500-nt DNA fragments and analyzed by ChIP assay.

In situ cross-linked TNF products containing the wild-type cells exposed to 39.5 °C showed that HSF-1 knockout mice demonstrated exaggeration of expression during exposure to febrile temperatures. This hypothesis was further supported by the studies of Xiao et al.† that HSF-1 might act as a repressor of macrophage TNF expression following challenge with LPS compared with HSF-1-sufficient littermates. We previously showed that HSF-1 binds to the murine TNFα proximal promoter/5′-UTR sequence spanning nt −85 to +138 under EMSA conditions.

We have extended these findings in our present study by localizing the HSF-1 binding site between nt +30 and 68 in the 5′-UTR of the murine TNFα gene. The sequence between nt +49 and +58, AGAAACATCCTTT, with the exception of a simple inversion of the 3′-terminal CT dinucleotide (underlined), is the canonical HSF-1 binding sequence. We used EMSA analysis to show that this sequence specifically binds HSF-1 and that mutation of the GAAan pentanucleotide at nt +49/+53 prevents binding of HSF-1, whereas the same substitution in a downstream GAAan sequence (nt +60/+62) had no effect on HSF-1 binding. However, the inability of DNA-protein com-

**FIG. 5.** EMSA analysis of HSF binding to wild-type and mutated TNFα fragment −85/+138. Nuclear extracts from Raw 264.7 cells exposed to 39.5 °C for 1 h were used as a source of HSF-1. PCR products containing the wild-type TNFα −85/+138 sequence (lane 1) or with GAA to CCC sequences at nt +49 (lane 2) were end-labeled and used as EMSA probes. Competition with a 100-fold molar excess of unlabeled +30/+68 wild-type (lane 3) and 49_Mut (lane 4) oligonucleotides was measured. The complex binding to the wild-type probe was supershifted with anti-human HSF-1 (lane 5), and the supershifted complex is represented by the arrowhead.

**FIG. 6.** Analysis of in vivo interactions between HSF-1 and the TNFα gene. Raw 264.7 cells were incubated at 39.5 °C for 60 min, cross-linked in situ with 1% (v/v) formaldehyde, sonicated to generate approximately 500-nt DNA fragments and analyzed by ChIP assay. Immunoprecipitates were analyzed by PCR using primers specific for the HRE-containing HSF70 promoter sequence (lanes 1–5) or the TNFα sequence between −85 and +138 containing the putative HSF-1 binding site (lanes 6–10). Samples were immunoprecipitated either without antibody (lane 5, 10), with an anti-HSF-1 antibody from two different sources, Stressgen (H1, lanes 3, 8) or Santa Cruz Biotechnologies (H2, lanes 4, 9), or with an irrelevant anti-human IL-13 antibody (lanes 2, 7). Lane 11 contains a 100-bp ladder. Lanes 1–6 contain the product of the PCR reaction with plasmid containing either the HSF70 promoter or the TNFα gene, respectively, added as template.

**FIG. 7.** Effect of HSF-1 overexpression on activity of reporter constructs driven by wild-type or nt +49-mutated TNFα promoter/5′-UTR. The wild-type (W/T) TNFα gene fragment −85/+138 (A), −244/+138 (B), or −1080/+138 (C) and comparable fragments with a GAA to CCC mutation at nt +50/−52 (MUT) were introduced into the PGL3 luciferase reporter construct and cotransfected into Raw 264.7 cells with the control plasmid pRL-SV40 alone or along with the indicated amount of HSF-1 expression plasmid. Cells transfected without the HSF-1 transfection plasmid were incubated at either 37 °C or 39.5 °C. All cells were stimulated with 100 ng/ml LPS and harvested 6 h later. The ratio of experimental to control luciferase activity was calculated, and data were calculated as percentage of control values in cells cultured at 37 °C without HSF-1. Mean ± S.E.; n = 6. *, p < 0.05 versus 0 HSF-1; †, p < 0.05 versus comparably treated wild-type-transfected cells.
plexes to form under EMSA conditions does not necessarily exclude an interaction in vivo. To further evidence that HSF-1 might bind to and repress the TNFα gene in vivo, we used the ChIP assay to determine if HS-1 interacts with the TNFα gene in the living cell. We incubated Raw 264.7 cells for 1 h at 39.5 °C, conditions that we have shown activate HSF-1 to a form that binds HS70 promoter and TNFα gene sequences under cell-free EMSA conditions. We showed that two different anti-HSF-1 antibodies communoprecipitated both HSα-containing HS70 promoter sequence and TNFα sequence containing the putative HS1-binding site. Negative controls, immunoprecipitated without antibody or with an irrelevant antibody (anti-IL-13) from the same species (rabbit), did not contain detectable HS70 or TNFα gene fragments demonstrating the specificity of the technique.

In transient transfection studies, the TNF–85+/+138 reporter construct containing a GAA to CCC substitution at nt +50/+52 was significantly less sensitive to the inhibitory effect of overexpressed HS-1 than was the wild-type construct. In fact, cotransfection of this mutated construct with 1 or 2 µg of HS-1 expression plasmid had no inhibitory effect on luciferase activity whereas the wild-type construct was inhibited by 30–40% when cotransfected with the same amount of HS-1 expression plasmid. These data suggest that HS-1 represses transcription from the minimal TNFα promoter/5′-UTR fragment, in part, by binding to the HRE-like sequence at +49/+58 nt within the 5′-UTR. The capacity of elements within the 5′-UTR region of genes to repress transcription has been reported to occur in other genes, including the collagen α1 gene and the potassium channel Kv3.1 gene (26, 27). Based on the location of the putative repressor site in the murine TNFα gene, only 30–60 nt downstream of the transcription start site, HS-1 might repress TNFα transcription by blocking RNA polymerase processivity, as has been shown in the phase T7 model system in which binding of the lac repressor 13–15 nucleotides downstream of the initiation site blocks T7 RNA polymerase processivity (28).

Interestingly, cotransfection with 3 µg of HS-1 or incubation at 39.5 °C caused comparable reductions in activity of the mutated and wild-type TNF–85+/+138 reporter constructs. The capacity of higher HS-1 levels to repress the TNF–85+/+138 construct containing the mutation at +50/+52 suggests that there might be additional pathways through which HS-1 represses expression of the minimal TNFα promoter. These include lower affinity binding of HS-1 to non-canonical HS-1 binding sites elsewhere in this gene fragment that is not detected by EMSA yet still might block transcription complex formation. Alternatively, HS-1 might repress TNFα transcription without directly binding to DNA. Chen et al. (22) have suggested that HS-1 could interact either with an upstream signal transduction component or with a coactivating factor to specifically repress genes such as c-fos and urokinase-type plasminogen activator.

In contrast to the minimal TNFα promoter, the full-length 1.2-kb TNFα promoter construct was comparably inhibited by 1–3 µg of HS-1, and this inhibition was unaffected by the presence or absence of the GAA to CCC mutation at +50/+52. The intermediate length construct, TNF–244/+138, exhibited a third pattern of responsiveness to the GAA to CCC mutation at +50/+52 and to overexpression of HS-1. The activity of the wild-type and mutated TNF–244/+138 was comparably inhibited by the lowest HS-1 expression but, when cotransfected with 2 and 3 µg of HS-1 expression plasmid, the wild-type construct was inhibited to a greater extent than the mutated construct. On the other hand, exposure to 39.5 °C reduced the activity of all three constructs, and the repression was unaffected by mutational inactivation of the HS1-binding site at nt +49/+58.

The different patterns of response of each of the three constructs to HS-1 overexpression and the mutational inactivation of the HS-1 binding site at nt +49/+58 suggest that additional sequences upstream of nt −85 might mediate transcriptional repression by HS-1. Using overlapping ~200-nt PCR-generated fragments of TNFα upstream sequence, the sequence upstream of nt −85 was scanned for HS-1 binding. Complex formation with rHSF-1 was detectable to the regions −1080/−845, −533/−196, and −326/−39 suggesting that redundant HS-1 binding sites might be active in the TNFα gene. Alternatively, or in addition to direct binding to DNA, HS-1 might interfere with transcription by binding to transcriptional activators as has been shown for STAT (29, 30), TFII D, and related factors (31).

In summary, we identified a unique HRE-like sequence in the murine TNFα 5′-UTR that binds HS-1 and is required for HS-1-mediated transcriptional repression in the minimal mouse TNFα promoter. This offers proof of the concept that HS-1 can repress gene transcription by binding to the 5′-UTR. Although the 5′-UTR HRE-like sequence of the mouse TNFα gene is not present in the human TNFα 5′-UTR, the results of this study suggest a number of additional redundant pathways through which exposure to febrile range temperature might inhibit transcription of both murine and human TNFα.

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REFERENCES

1. Beutler, B. (1995) J. Invest. Med. 43, 227–235
2. Chollet-Martin, S., Montravers, P., Gilbert, C., Elbim, C., Elblin, C., Desmonte, J. M., Fagun, J. Y., and Gougerot-Pocidalo, M. A. (1993) Am. Rev. Respir. Dis. 146, 990–996
3. Beutler, B., Milsark, I., and Cerami, A. (1985) Science 229, 869–871
4. Cross, A. S., Sadoff, J. C., Kelly, N., Bernston, E., and Gemski, P. (1989) J. Exp. Med. 169, 2021–2027
5. Cross, A., Ashley, L., Seguin, M., Yuan, L., Kelly, N., Hambuck, C., and Sadoff, J. (1995) J. Clin. Invest. 96, 676–686
6. Saribah, E., Imamura, K., Luehrs, R., and Kufe, D. (1988) J. Clin. Invest. 81, 1506–1510
7. Jongsma, C. V., Shakhov, A. N., Nedosposav, S. A., and Cerottini, J.-C. (1989) Eur. J. Immunol. 19, 549–552
8. Biragyn, A., and Nedosposav, S. A. (1995) J. Immunol. 155, 674–683
9. Lieberman, A. P., Pitha, P. M., and Shin, M. L. (1990) J. Exp. Med. 172, 989–992
10. Ensor, J. E., Crawford, E. K., and Hasday, J. D. (1999) Am. J. Physiol. 268, C1140–C1146
11. Beutler, B., Krochin, N., Milsark, I. W., Luedke, C., and Cerami, A. (1986)
12. Ensor, J. E., Wiener, S. M., McCrea, K. A., Viscardi, R. M., Crawford, E. K., and Hasday, J. D. (1994) *Am. J. Physiol.* **266**, C967-C974
13. Jiang, Q., DeTolla, L., Kalvakolanu, I., Van Rooijen, N., Singh, I. S., Fitzgerald, B., Cross, A. S., and Hasday, J. D. (1999) *Infect. Immun.* **67**, 1539–1546
14. Jiang, Q., DeTolla, L., Kalvakolanu, I., Fitzgerald, B., and Hasday, J. D. (1999) *Am. J. Physiol.* **276**, R1653–R1660
15. Singh, I. S., Calderwood, S., Kalvakolanu, I., Viscardi, R., and Hasday, J. D. (2000) *J. Biol. Chem.* **275**, 9841–9848
16. Fairchild, K. D., Viscardi, R. M., Hester, L., Singh, I. S., and Hasday, J. D. (2000) *J. Interferon Cytokine Res.* **20**, 1049–1055
17. Sorger, P. K. (1991) *Cell* **65**, 363–365
18. Sarge, K. D., Murphy, S. P., and Morimoto, R. I. (1993) *Mol. Cell. Biol.* **13**, 1392–1407
19. Cotto, J. J., Kline, M., and Morimoto, R. I. (1996) *J. Biol. Chem.* **271**, 3355–3358
20. Periasy, O., Xiao, H., and Liu, J. T. (1998) *Cell* **90**, 787–796
21. Cahill, C. M., Warerman, W. R., Xie, Y., Auron, P. E., and Calderwood, S. K. (1996) *J. Biol. Chem.* **271**, 24874–24879
22. Chen, C., Xie, Y., Stevenson, M. A., Auron, P. E., and Calderwood, S. K. (1997) *J. Biol. Chem.* **272**, 26803–26806
23. Schrinner, K., Matthias, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17**, 6419
24. Wu, B. J., Kingston, R. E., and Morimoto, R. I. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 629–633
25. Xiao, X., Zou, X., Davis, A. A., McMillan, D. R., Curry, B. B., Richardson, J. A., and Benjamin, I. J. (1999) *EMBO J.* **18**, 5943–5952
26. Hernandez, I., de la Torre, P., Rey-Campos, J., Garcia, I., Sanchez, J. A., Munoz, R., Rippe, B. A., Munoz-Yague, T., and Solis-Herruzo, J. A. (2000) *DNA Cell Biol.* **19**, 341–352
27. Gan, L., Hahn, S. J., and Kaczmarek, L. K. (1999) *J. Neurochem.* **73**, 1350–1362
28. Lopez, P. J., Guillerez, J., Sousa, R., and Dreyfus, M. (1998) *J. Mol. Biol.* **276**, 861–875
29. Stephanou, A., Isenberg, D. A., Nakajima, K., and Latchman, D. S. (1999) *J. Biol. Chem.* **274**, 1723–1728
30. Stephanou, A., and Latchman, D. S. (1999) *Gene Expr.* **7**, 311–319
31. Yuan, C. X., and Gurley, W. B. (2000) *Cell Stress Chaperones* **5**, 229–242