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The Ewing Sarcoma Protein (EWS) Binds Directly to the Proximal Elements of the Macrophage-Specific Promoter of the CSF-1 Receptor (*csf1r*) Gene¹

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Many macrophage-specific promoters lack classical transcriptional start site elements such as TATA boxes and Sp1 sites. One example is the CSF-1 receptor (CSF-1R, CD115, *c-fms*), which is used as a model of the transcriptional regulation of macrophage genes. To understand the molecular basis of start site recognition in this gene, we identified cellular proteins binding specifically to the transcriptional start site (TSS) region. The mouse and human *csf1r* TSS were identified using cap analysis gene expression (CAGE) data. Conserved elements flanking the TSS cluster were analyzed using EMSAs to identify discrete DNA-binding factors in primary bone marrow macrophages as candidate transcriptional regulators. Two complexes were identified that bind in a highly sequence-specific manner to the mouse and human TSS proximal region and also to high-affinity sites recognized by myeloid zinc finger protein 1 (Mzf1). The murine proteins were purified by DNA affinity isolation from the RAW264.7 macrophage cell line and identified by mass spectrometry as EWS and FUS/TLS, closely related DNA and RNA-binding proteins. Chromatin immunoprecipitation experiments in bone marrow macrophages confirmed that EWS, but not FUS/TLS, was present in vivo on the CSF-1R proximal promoter in unstimulated primary macrophages. Transfection assays suggest that EWS does not act as a conventional transcriptional activator or repressor. We hypothesize that EWS contributes to start site recognition in TATA-less mammalian promoters. *The Journal of Immunology*, 2008, 180: 6733–6742.

Macrophages are a specialized lineage of hematopoietic cells with a distinct gene expression profile (1) required to fulfil their many roles in innate immunity (2). Their proliferation, differentiation, and survival are dependent on the actions of the growth factor macrophage CSF-1, which acts by binding to a specific receptor (CSF-1R, CD115) encoded by the *csf1r* (*c-fms*) protooncogene. CSF-1R protein expression is restricted to cells of the macrophage lineage, and a *csf1r*-EGFP transgene provides a unique marker for cells of this lineage in mice (3). Consequently, the transcriptional regulation of the *csf1r* gene has been widely studied as a model for understanding the nuclear events underlying macrophage lineage commitment (2, 4, 5).

The proximal promoter of the *csf1r* gene used in macrophages is an archetype for a class of mammalian promoters that lacks conventional proximal promoter elements such as a TATA box,

CCAAT box, or GC-rich elements bound by the transcription factor Sp1 (6, 7). Instead, it contains multiple copies of purine-rich sequences recognized by members of the Ets transcription factor family, notably the macrophage-specific transcription factor PU.1 (2). PU.1 is able to bind directly to components of the basal transcription machinery such as TATA-binding protein (8–11), and a multimerized PU.1 recognition motif can generate a minimal macrophage-specific promoter (12). The activity of this artificial promoter requires cooperation between PU.1 and another member of the Ets family (12). Nevertheless, such a promoter is very weakly active compared with the native *csf1r* proximal promoter in transfections of macrophage cells, so we considered that PU.1 alone is probably not the only DNA-binding protein required for start-site specification in myeloid promoters. Furthermore, the functions of a basal promoter are more complex than simple transactivation or transrepression and include functions such as polymerase recruitment and activation, transcription initiation and termination, the regulation of splicing, histone positioning, and chromatin conformation. In an attempt to further our understanding of the control of *csf1r* transcription, we looked for additional proteins that bind specifically to the start-site region of the *csf1r* promoter.

In this paper, we have identified protein complexes that bind related elements of the start-site region of both the mouse and human *csf1r* promoters.

Materials and Methods

Cell culture

Cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated FBS (BioWhittaker) and L-glutamine (2 mM, GlutaMAX), 30 U/ml penicillin, and 100 μ g/ml streptomycin (all from Invitrogen). Cells were maintained in a humidified tissue culture incubator at 37°C in the presence of 5% CO₂.

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Murine bone marrow-derived macrophages (BMM)³ were obtained by differentiation of mouse bone marrow cells in the presence of 10⁴ U/ml human recombinant CSF-1 (Chiron). Briefly, adult mice (BALB/c or C57BL/6) were culled by cervical dislocation and an incision was made along the inner thigh of the hindlimbs. Each femur was exposed and resected, after which the bone was sterilized and cleaned with 70% ethanol. Dissected femurs were opened at both ends and the marrow cells were flushed out with complete RPMI 1640 medium by the use of 27-gauge needles fitted on 10 ml syringes. Cell clumps were disaggregated by pipetting up and down several times, and cell suspensions were grown in tissue culture medium in the presence of CSF-1. On day 3 the medium was changed and the culture was continued up to 7 days when typically >95% of cells are macrophages (13). RAW264.7 cells were obtained from the American Type Culture Collection.

Nuclear extraction

Nuclear extracts were prepared using a variation of the method described by Osborn et al. (14). All solutions used were ice-cold and contained either Complete Mini Protease Inhibitor Tablet (Roche Applied Science) or a combination of 0.5 mM PMSF, 1 mM DTT, 1 µg/ml aprotinin, and 1 µg/ml leupeptin. For bulk preparation, adherent cells from six 10-cm-diameter TC dishes were harvested and pooled in 50 ml polypropylene centrifuge tubes. Cells were then pelleted at 400 × *g* for 5 min at 4°C and washed once with ice-cold PBS and once with hypotonic wash buffer (buffer A) comprising 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 2 mM MgCl₂, and 0.1 mM EGTA. Washed cells were pelleted, resuspended in 1 ml buffer A, and transferred to 10 ml tubes.

A 2-ml aliquot of cell lysis buffer (buffer B; 25 mM HEPES (pH 7.9), 10 mM KCl, 0.5 mM EGTA, 2 mM MgCl₂, 0.2% Nonidet P-40) was added at 4°C, mixed, and the nuclei were pelleted gently at 250 × *g* for 10 min at 4°C. Supernatants were removed and nuclei were extracted by resuspending in 500–750 µl of nuclear extraction buffer (buffer C; 20 mM HEPES (pH 7.9), 420 mM NaCl, 20% glycerol, 0.2 mM EDTA, 0.2 mM EGTA). Nuclei were incubated in this buffer for 15 min on ice with gentle shaking, followed by centrifugation of the extracts at 10,000 × *g* for 10 min at 4°C. Supernatants were removed carefully, snap frozen in an ethanol/dry ice bath, and stored at –70°C.

For magnetic DNA affinity purification of DNA-binding proteins (see below), large-scale nuclear extractions were performed from RAW264.7 cells. Typically, for one preparation, cells were harvested from 10 to 12 Sterilin 10 × 10 cm TC dishes. Depending on the number of cells obtained, buffer A used was 4–5 ml, while buffers B and C were 2.5 and 1 ml, respectively.

EMSA

Oligonucleotide probes for EMSA were prepared by end labeling 5 µM oligonucleotides with [^γ-³²P]ATP (10 mCi/ml, 3000 Ci/mmol) using T4 polynucleotide kinase (New England Biolabs) in 1 × polynucleotide kinase buffer for 30 min at 37°C. Unincorporated radionucleotides were removed by passing the reaction through a Sephadex G25 column (NICK column, Pharmacia). Radiolabelled probes were obtained by collecting 5-drop (~200 µl) fractions. The radioactivity of the probe was monitored using a Geiger-Müller counter, and the most radioactive void volume fractions were then used in gel-shift experiments. Based on the shape of the elution curve and the known amount of oligonucleotide loaded, the concentration of probe in the peak fractions was estimated, assuming 100% recovery of the oligonucleotides in the void peak.

Binding of nuclear proteins to the oligonucleotide was performed by incubating 2 µg or a 2-µl aliquot of nuclear extracts, 0.5 µg of poly(dI:dC)(dI:dC) (Amersham Pharmacia Biotech), and 0.5 ng of end-labeled double-stranded oligonucleotides in buffer containing 15% glycerol, 40 mM KCl, 20 mM HEPES (pH 7.9), 5 mM DTT, and 2 mM EDTA. The reaction was incubated for 30 min at room temperature (RT). Supershift experiments used 1 µl of either anti-Ewing sarcoma (EWS) Ab (Santa Cruz Biotechnology) or anti-FUS Abs, which were kindly provided by Dr. Mark C. Alliegro (Department of Cell Biology and Anatomy, LSU Health Sciences Centre, New Orleans, LA). Protein-probe complexes were separated on a discontinuous 4–8% polyacrylamide gel system (29/1 polyacrylamide, 0.25 M Tris (pH 8.8)) in a Bio-Rad Mini Protean apparatus with running buffer containing 25 mM Tris, 200 mM glycine, and 0.2 mM EDTA. Samples were electrophoresed at 100 V until the loading dye

reached the bottom of the gel. Subsequently, gels were removed from the apparatus, fixed in 10% acetic acid, dried into filter papers in a Bio-Rad gel dryer, and exposed into Fuji Super RX X-Ray film for autoradiography.

Magnetic DNA affinity purification of *csf1r* promoter binding proteins

RAW264.7 nuclear extract was diluted with binding buffer (10 mM HEPES, 2 mM DTT, 0.5 µg/ml aprotinin, 0.5 µg/ml leupeptin, 10% glycerol, 0.5 mM PMSF) to mimic the binding conditions used in EMSA/gel-shift experiments. Subsequently, nonspecific protein-DNA binding was blocked by addition of poly(dI:dC)(dI:dC) at 500 ng/ml and incubation for 5 min at RT. Meanwhile, streptavidin-coated paramagnetic beads (Dynabeads from Dynal Biotech, MagneSphere from Promega, or streptavidin magnetic particles from Roche Applied Science) were washed from their storage solutions with 1 × binding buffer.

HPLC-purified biotinylated oligonucleotides were ordered from GeneWorks. Before binding, biotinylated oligonucleotides (sense strands) were annealed with their antisense strands by heating them at 65°C for 10 min and incubating at RT for at least 10 min. Double-stranded oligonucleotides were then bound to washed streptavidin-coated magnetic beads, with a ratio of 1 mg particles to 120 µM biotinylated oligonucleotides. Binding was performed in 10 mM Tris-HCl at RT for at least 15 min with gentle agitation in a rotary shaker. After binding, excess oligonucleotides were washed away with 1 × binding buffer before addition of nuclear extract.

DNA-binding protein isolation was performed by incubating the pre-cleared nuclear extracts with the immobilized biotinylated oligonucleotides in 15 ml tubes for 1 h at RT with gentle rotation of tube in rotary shaker. After binding, reactions were transferred into 2 ml tubes and placed in magnetic particle separator to capture the magnetic bead-oligonucleotide-protein complex, and washed with binding buffer containing 50 mM NaCl and 30 mM KCl. Four 1 ml washes were performed, and the bound proteins were eluted with 200 µl of 300 mM ammonium acetate (pH 4.2). Second elutions were performed with 200 µl of water. The eluted proteins were concentrated by diluting the samples into 50 mM ammonium acetate with MilliQ water followed by concentration in Microcon YM30 spin columns (Millipore).

SDS-PAGE

Proteins were electrophoretically resolved using SDS-PAGE typically at a gel concentration of 12%. The Bio-Rad Mini Protean apparatus was used according to the manufacturer's instructions. In some cases, proteins were separated in precast 12% NuPAGE Bis-Tris gels (Invitrogen) in XCell SureLock MiniCell apparatus (Invitrogen) using NuPAGE MOPS SDS running buffer. The method used was as described by the manufacturer. After separation, gels were fixed and stained with Coomassie brilliant blue R250 (Sigma-Aldrich) diluted in methanol-water-glacial acetic acid (45/45/10) for 30 min, followed by destaining of excess dye in 30% methanol-10% acetic acid.

In-gel trypsin digestion of protein bands and mass spectrometry

For mass spectrometry analysis, protein bands of interest and blank gel for controls were excised from the gel with sterile scalpel blades ready for subsequent steps.

Excised gel slices were dehydrated in 100% methanol for 5 min followed by rehydration in 30% methanol for 5 min. Rehydrated gel slices were subsequently washed twice in ultrapure water for 10 min each. Each gel band was then washed three times for 10 min each with 100 mM ammonium bicarbonate (pH 7.5) containing 30% acetonitrile. After the last wash, the gel was cut or crushed into small pieces, washed in ultrapure water, and vacuum dried in a vacuum centrifuge for 30 min. Gel pieces were then resuspended in 50 mM ammonium bicarbonate (pH 7.5) containing 5–10 ng/µl sequencing-grade trypsin (Promega) and incubated overnight for 24 h at 37°C. The following day, supernatant was transferred into fresh tubes and the remaining peptides in the gel were extracted with 50% acetonitrile containing 0.1% trifluoroacetic acid. This was combined with the supernatant ready for mass spectrometry analysis, ensuring that the final acetonitrile concentration was ≤5%.

A Hewlett-Packard 1100 HPLC system was used for liquid chromatography separation. An aliquot (10 µl) of peptide digest was injected onto a Zorbax C₁₈ reversed-phase HPLC column (2 mm internal diameter). Tryptic peptides were eluted at 0.3 ml/min with a 0–45% gradient of acetonitrile with 0.1% TFA. The output stream was split ~1:10 and peptides were analyzed during elution by electrospray ionisation (ESI) quadrupole and time-of-flight mass spectrometry (qTOF) in independent data acquisition mode. Fractions were also collected and where required were loaded into

³ Abbreviations used in this paper: BMM, bone marrow-derived macrophage; CAGE, cap analysis of gene expression; CHIP, chromatin immunoprecipitation; EWS, Ewing sarcoma protein; FANTOM, functional annotation of mouse transcriptome project; Inr, initiator; MS/MS, tandem mass spectrometry; RT, room temperature; TSS, transcriptional start site.

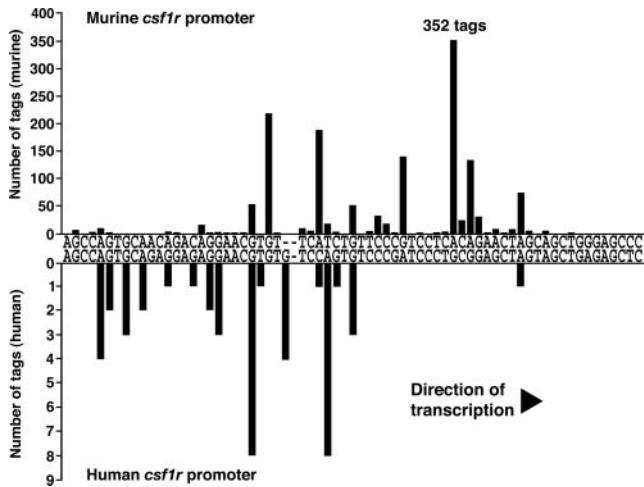


FIGURE 1. CAGE tag analysis of murine and human *csf1r* proximal promoters. The number of tags identified at a particular position corresponds to the frequency of initiations at that position. The sequence shown begins at -120 (murine) or -122 nucleotides (human) relative to the ATG codon.

nanospray needles for analysis by direct nanospray ESI-qTOF mass spectrometry to obtain reliable peptide sequence for de novo sequence analysis.

Primary peptide sequence was deduced from tandem mass spectrometry, and the National Center for Biotechnology Information (NCBI) protein sequence database was queried using the resultant peptide sequences to identify candidate proteins with pBLAST (www.ncbi.nlm.nih.gov/BLAST/).

Transient transfection analysis

The preparation of transient and stable transfectants was conducted as previously described (15). Briefly, transfection was achieved by electroporation of 5×10^6 cells in $400-450 \mu\text{l}$ of RPMI 1640 medium buffered with 20 mM HEPES (pH 7.4) using a Gene Pulser electroporator (Bio-Rad) set at 280 V with a capacitance of $960 \mu\text{F}$. For transient transfection, $10 \mu\text{g}$ of

reporter plasmid was used, with $1 \mu\text{g}$ of expression vector or control vector (pEF6). For stable transfection, $8 \mu\text{g}$ of reporter plasmid was used with $2 \mu\text{g}$ of pNPT-neo (which confers resistance to neomycin). Immediately following electroporation, cells were diluted into complete medium, pelleted, washed, and replated. For transient transfections, cells were harvested at 24 h for luciferase assays by briefly washing adherent cells with PBS followed by lysis in 1 ml luciferase lysis buffer containing 500 mM HEPES, 1 mM MgCl_2 , 1 mM DTT, and 0.2% Triton X-100 detergent. The cellular debris was pelleted at $12,000 \text{ rpm}$ and the supernatant retained for assay using the LucLite reporter gene assay kit (Packard) according to the manufacturer's instructions. Light emission was measured with a Packard TriLux luminometer and the output expressed as relative light units (RLU). The protein concentrations of the lysate supernatants were measured with the Bio-Rad protein assay using the manufacturer's protocol.

Plasmid reporter constructs used in transfection analysis

Luciferase reporter constructs (0.3 , 0.5 , and 6.7 kb *csf1r*-luciferase) comprising portions of the murine *csf1r* promoter in pGL2 (Promega) were as previously described (15, 16). Both substitution and deletion mutations of the *csf1r* promoter were made in 0.5-kb *csf1r*-luciferase by splice overlap PCR (17). The resultant plasmids were sequenced to check that the mutations had been correctly made.

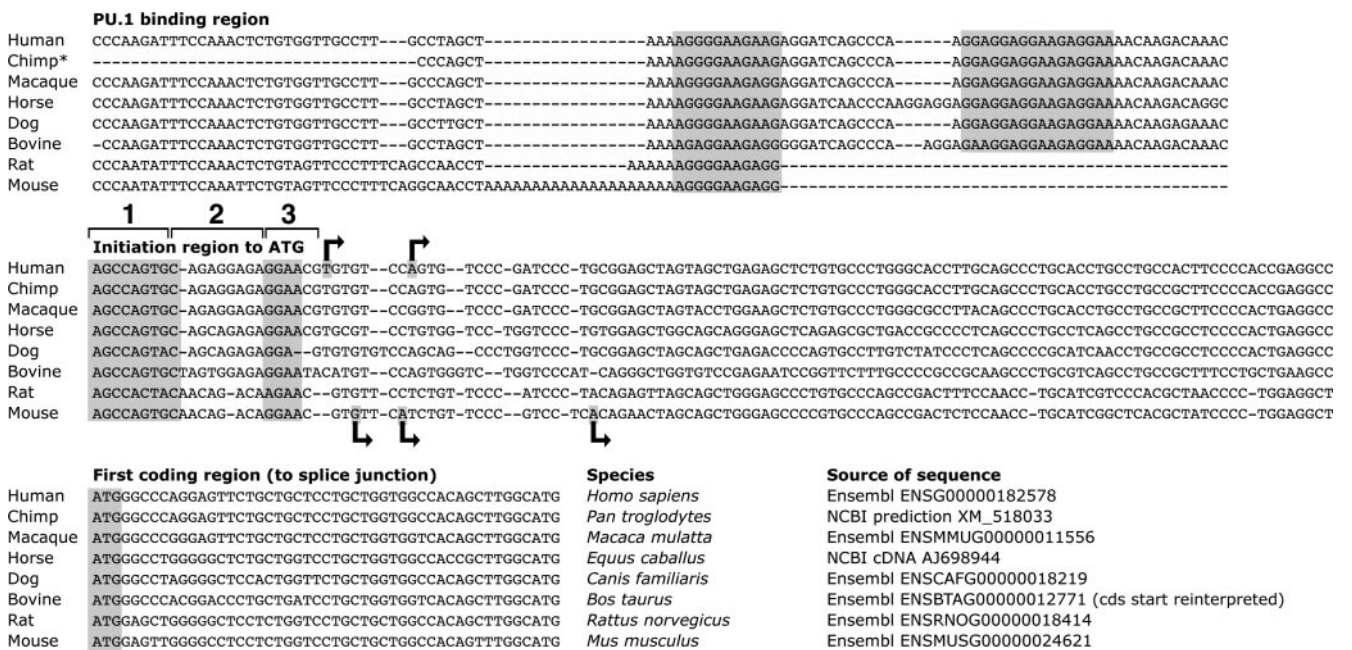
Plasmid expression constructs used in transfection analysis

Expression constructs for murine EWS and FUS/TLS were made in the pEF6 vector (Invitrogen) by PCR amplification of murine cDNA using the following primer sets: EWS: forward primer, $5'\text{-GAAGGGCGAGAAAATGGCGTC-3}'$; reverse primer, $5'\text{-GTAGGGCCGGTCTGCGTT-3}'$ (which excludes the natural stop codon so that the C-terminal V5-His tag is enabled); FUS/TLS: forward primer, $5'\text{-TGCGGGACATGGCTTCAA-3}'$; reverse primer, $5'\text{-ATATGGCCTCTCCCTGCGATCCT-3}'$.

Expression constructs in pEF6 were checked by sequencing to ensure that they were correct.

Chromatin immunoprecipitation (ChIP) experiments

ChIP assays were performed as described earlier (18–20). Approximately 5×10^5 cell equivalents (one sixth) of the sheared soluble chromatin was precleared with tRNA-blocked protein G agarose, and 10% of the pre-cleared chromatin was set aside as an input control. Immunoprecipitation was conducted with $5 \mu\text{g}$ of Abs overnight at 4°C . The PU.1 Ab has been



*The first base pairs listed here from the chimpanzee cDNA sequence mark a splice site so the remainder of the 5' cDNA sequence is irrelevant. The *Pan troglodytes* genomic sequence in Ensembl appears to be missing at this point, so the predicted cDNA is presently the only available sequence.

FIGURE 2. Alignment of the *csf1r* transcriptional start region in several vertebrate species. Only the major start sites are shown for human and mouse genes. The shaded regions in the PU.1 binding region correspond to experimentally verified PU.1 binding sites (in mice and humans). In the initiation region, shaded sections (regions 1–3) correspond to the EWS/TLS binding region, including the conserved AGCCAGTGC and GGAA motifs. The ATG initiation codon in the first coding region is also shaded.

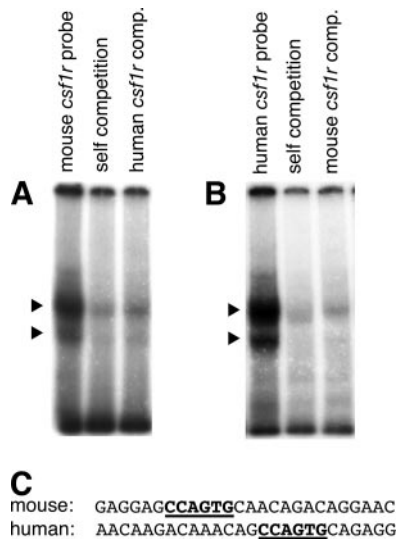


FIGURE 3. A nuclear DNA binding complex is observed for both murine and human *csflr* promoter sequences. EMSAs of the murine (A) and human (B) promoter sequence are shown immediately upstream of the main transcriptional initiation sites using BMM nuclear extract. The highly conserved CCAGTG motif is shown in boldface type (C). A pair of protein-oligonucleotide complexes with identical mobility are present with both murine and human oligonucleotides (arrowed). These complexes are competed by cold self oligonucleotide (100× molar excess) and are cross-competed by the alternate species, indicating that this region of the human and murine promoter sequences binds the same protein(s).

described earlier (18). EWS Ab was purchased from Santa Cruz Biotechnologies, and rabbit polyclonal FUS Ab was purchased from Bethyl Laboratories. Immune complexes were pulled down using protein G agarose and washed, decrosslinked, and purified as described earlier (18).

Samples were analyzed by real-time PCR with a probe sequence derived from the Roche universal probe library (Roche Diagnostics) using the Fast-Start TaqMan master kit (Roche). Primers for the probe were 5'-GGGCA GATGAGAAAGGTATGA-3' (forward) and 5'-AGTCTCCAGAT GAGCAGTGA-3' (reverse), which generate a 77-bp amplicon across the *csflr* promoter. The thresholds for the promoters being studied were adjusted using input threshold values as reference values and are represented as relative enrichment.

Results

Determination of the *in vivo* start site in the mouse and human *csflr* genes

Recent data produced by the FANTOM3 (functional annotation of mouse transcriptome project 3) consortium has provide a unique resource for precise annotation of start site usage in the mouse and human genes based upon cap analysis gene expression (CAGE) technology (6) (fantom.gsc.riken.go.jp). In both mouse and human *csflr* genes, transcription initiates in a relatively broad region covering 30–50 bp. The transcription start site (TSS) in both genes was previously determined using reverse transcriptase primer extension or RNase protection, neither of which had perfect base pair accuracy or the quantitative data to identify relative start usage in a range of conditions. For the CAGE analysis in the mouse, some 20 separate libraries derived from BMMs cultured with a range of stimuli were polled. A comparative analysis of the CAGE tag distribution for the mouse and human promoter regions is shown in Fig. 1. The data for the mouse are not completely consistent with previous data based on primer extension (16), probably due to the use of a primer that was too close to the 5' end based upon the longest cDNA sequence then available. However, the original data based upon RNase protection (16) was found to be completely consistent with the CAGE pattern shown. Close examination of the

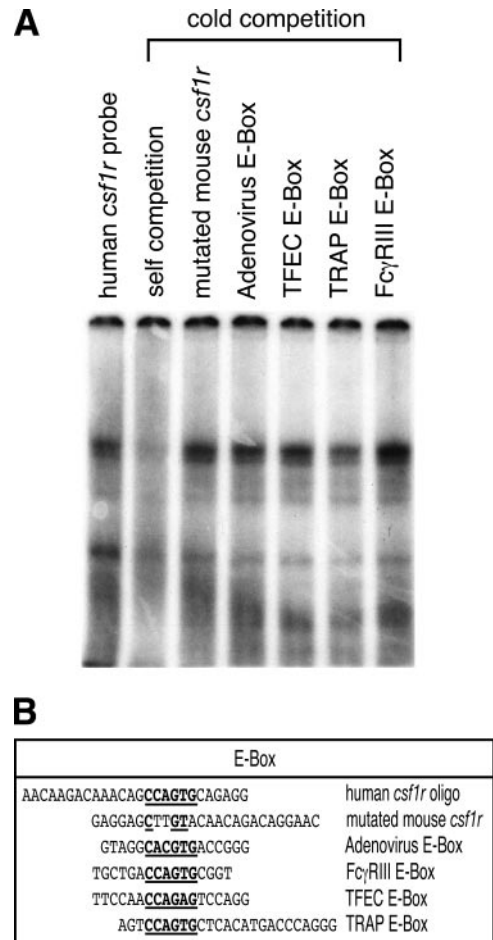


FIGURE 4. The conserved *csflr* promoter region is not an E box. A, EMSA of labeled human *csflr* oligonucleotide in the presence of cold competitors comprising a series of oligonucleotides containing E box motifs similar to the highly conserved CCAGTG motif in the *csflr* promoter. Using BMM nuclear extract, the previously observed *csflr* promoter binding complexes are effectively competed by a 100× molar excess of self oligonucleotide. B, Although a mutation in the putative E box (CCAGTG→CTTGTA) in the murine *csflr* promoter abolishes competition by the murine sequence, a series of known E box motifs at 100× molar excess also fail to compete, suggesting that the observed complexes are not due to known E box binding proteins present in macrophages.

major start sites reveals that they all conform to pyrimidine/purine initiator core sequence, mostly the preferred CA, as in most mammalian promoters (6). The sequence surrounding this is more variable; that is, no site conforms to the initiator (Inr) consensus YYANWYY, although the major start site in mice and one of the human major sites conform in at least four of the six positions.

A comparison of promoters from different species (Fig. 2) shows that the human sequence is ancestral and a deletion has occurred in the rodents that removes one PU.1 site. The deletion actually shifts the upstream PU.1 site into the same position (relative to the transcription start sites) as the one that was removed. The basic promoter structure is conserved throughout the mammals, and Follows et al. (21) have demonstrated that the overall chromatin organization of the murine and human promoters is also conserved. Even *Xenopus* (Ensembl ENSXETG00000011887) appears to have a similar structure including a purine-rich promoter and, based on the mRNA from NCBI (NM_001008180), a similar transcript initiation site to the murine and human promoters.

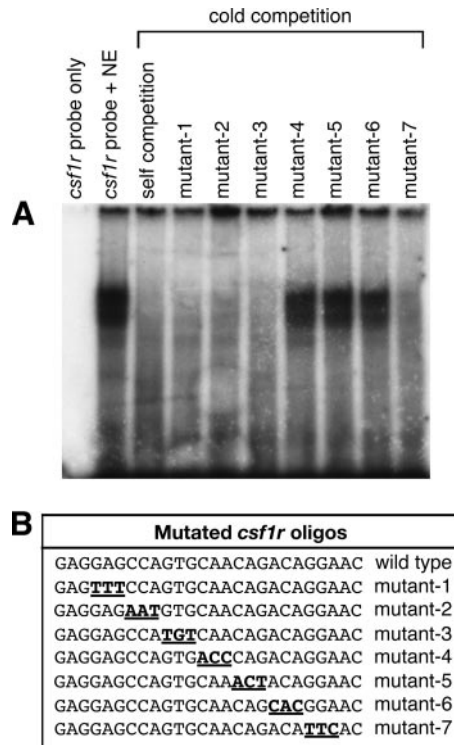


FIGURE 5. Mutational analysis reveals the binding site for the *csflr* binding complexes includes the region CAACAGACA. *A*, EMSA was conducted using labeled wild-type murine *csflr* oligonucleotide and BMM nuclear extract. Cold competition was conducted with oligonucleotides comprising a series of mutations across the sequence, extending farther than the conserved CCAAGTG motif. Only mutants 4, 5, and 6 failed to compete, suggesting that the sequence in this region (as well as the previously identified TT mutant) was important for binding. *B*, Sequences of the mutated oligonucleotides used for competition experiments.

Conserved motifs exist upstream of the transcription initiation sites

In earlier studies, we mutated the sequence CAGGAA from the promoter on the presumption that it was centrally placed within the TSS region. The mutation abolished proximal promoter activity in transfected macrophage cell lines (12). Tagoh et al. (22) showed that during macrophage differentiation from progenitors, this site becomes hypersensitive to modification by dimethyl sulfate as detected by in vivo footprinting. The site was mistakenly annotated as a PU.1 binding site, but it does not bind PU.1 in either EMSA or DNase protection assays (12). The CAGE (and RNase protection data) reveals that this motif is actually immediately upstream of the major start sites, where the preinitiation complex must assemble. The in vivo dimethyl sulfate footprinting data of Tagoh

et al. (22) actually revealed macrophage-specific hyperreactive G(N7) sites extending from the CAGGAA motif upstream through the sequence GCCAGTGCAACAGACAGGAAA (Fig. 2). As well as the conserved Ets-like motif GGA(A/G), this upstream sequence contains a motif AGCCAGTG that is conserved between the murine and human promoters, as well as widely in other species (Fig. 2, region 1). This motif (which resembles an E box consensus CANNTG) is immediately (20–30 bp) upstream of the cluster of start sites in humans and mice in a region that might be considered to function like a TATA box. Given the evidence that the site is specifically occupied in macrophages, we set out to identify the proteins that bind to it.

EMSA of macrophage nuclear proteins binding the mouse and human *csflr* proximal promoter motifs

To determine whether macrophages contain nuclear DNA binding proteins that can bind the *csflr* proximal promoter element immediately adjacent to the TSS region, and to confirm the functional equivalence of the mouse and human proximal promoter regions, we performed EMSAs using nuclear extracts from murine BMM. As shown in Fig. 3, both the mouse and human elements bound to a broad doublet on EMSA that was cold-competed by either self or by the corresponding site from the other species. These cannot be Ets proteins. In considering candidate proteins that might bind this sequence, we examined the possibility that the CCAAGTG sequence of interest could constitute a variant E box, because macrophages express several members of the basic helix-loop-helix transcription factor family (23). Fig. 4 shows that several sequences from other myeloid promoters that contain this CCAAGTG sequence failed to cold compete against the human *csflr* probe; however, mutation of the core CCAAGTG to CTTGA in the mouse sequence reduced cold competitor activity, arguing that this motif does form part of the murine recognition sequence. We therefore searched across the entire mouse sequence using single base pair substitution, but did not identify any single substitution that abolished binding activity (data not shown). Fig. 5 shows the results of mutating bases from the murine *csflr* TSS-flanking element three at a time. Paradoxically, in this particular series, the substitutions chosen over the CCAAGTG motif (mutants 1–3) competed effectively, suggesting that these mutant oligonucleotides still bound the factor(s) concerned. In contrast, the next three downstream triplet substitutions (mutants 4–6) failed to compete. Finally, mutant 7 competes successfully, demonstrating that the binding site is confined to the region specified by mutants 4–6. Overall, this suggests that the protein(s) concerned require both the CCAAGTG (region 1) motif and the downstream CAACAGACA (region 2), but that the triplet substitutions made in the CCAAGTG motif still allow binding, unlike the TT-A substitution made earlier (Table I).

Finally, we noted a similarity between the human and mouse elements and the rather loose consensus of the so-called X box

Table I. EMSA competition by mutations around the conserved CCAAGTG motif^a

Oligonucleotide Sequence 5' to 3'	Experimental Result
GAGGAGCCAGTGCAACAGACAGGAAC	Wild type: competes
GAGGAGCTTTGTA CAACAGACAGGAAC	Fig. 4 mutant: fails to compete
GAGTTTCCAGTGCAACAGACAGGAAC	Fig. 5 mutant 1: competes
GAGGAGAAATGTGCAACAGACAGGAAC	Fig. 5 mutant 2: competes
GAGGAGCCATGTC AACAGACAGGAAC	Fig. 5 mutant 3: competes
GAGGAGCCAGTGACCCAGACAGGAAC	Fig. 5 mutant 4: fails to compete
GAGGAGCCAGTGCAAACTACAGGAAC	Fig. 5 mutant 5: fails to compete
GAGGAGCCAGTGCAACAGCACGGAAC	Fig. 5 mutant 6: fails to compete
GAGGAGCCAGTGCAACAGACATTTCAC	Fig. 5 mutant 7: competes

^a Conserved motif boxed; mutated base pairs are shaded gray.

Table II. X box sequences used in competition EMSA experiments against the human *csf1r* putative X box

Sequence	Origin
AACAAGACA AACAGCC AGTGCAGAGG	Human <i>csf1r</i> promoter ^a
NNG TNRCC NNR GYAAC NN	RFX consensus
TCCCTAGC AACAGAT GCGT	DRA X box
GATCTGA GTAGTTATGGTAACTG	MIE <i>c-myc</i> X box
GATCCGT TGCTCGGCAACGGCCTA	Hepatitis B X box
CTG CCCAGAAACAAGT GATG	Li promoter X box
CAGTGTGCCTAGGAGACAG	IL5 promoter X box
CCCTAG CAACA GATGCC	RFX1 consensus

^a Probe sequence against which competition was performed.

(24) that binds members of the RFX transcription factor family, but none of a series of known functional X box motifs (Table II) showed any ability to cold compete with the human probe (data not shown). Based on all of these findings, the binding activity of the macrophage nuclear proteins that recognize the mouse and human *csf1r* proximal promoters is highly sequence-specific and involves residues spaced over 9–12 bp pairs, any one of which can be substituted without dramatically reducing binding activity.

Purification and identification of the *csf1r* proximal promoter binding proteins

To directly identify the proteins that bind the TSS-flanking element of the proximal promoter of the *csf1r* promoter, we performed oligonucleotide affinity chromatography on RAW264.7

nuclear extracts, using murine *csf1r* oligonucleotides that were 5' biotinylated and immobilized on streptavidin-coated paramagnetic beads. Two major bands (apparent molecular masses of 70 and 110 kDa) were identified reproducibly on Coomassie-stained gels (Fig. 6A) with two separate, overlapping oligonucleotides, and were excised and subjected to protein identification by tandem mass spectrometry. Using de novo tandem mass spectrometry (MS/MS) sequencing in addition to peptide mass fingerprinting, the two proteins were identified unequivocally as FUS/TLS and the closely related EWS protein (Table III).

The use of overlapping but slightly displaced probes (containing regions 1 + 2 and 2 + 3) suggested that EWS binds preferentially to the distal (3') site (perhaps requiring some downstream sequence in addition to the G-rich region), while FUS/TLS binds equally well to both probes, presumably in the overlapping region. PU.1 bound only to the X box oligonucleotide that contains the known PU.1 site. To confirm the identity of these proteins as the major bands seen on EMSA, we performed Ab supershift experiments. Abs against either EWS or FUS/TLS generated a supershifted complex in macrophage nuclear extracts (Fig. 6B) and the amount of supershifted complex increased with the amount of nuclear extract added. EWS and FUS/TLS are both known primarily as RNA-binding proteins. To our knowledge, EWS has not previously been shown to bind to double-stranded DNA, but FUS/TLS was previously discovered as downstream target of the bcr-abl oncogene, based upon affinity purification using a consensus zinc finger oligonucleotide that is bound by the myeloid zinc finger protein Mzf-1 (25, 26). Accordingly, we performed EMSA using the same consensus zinc finger oligonucleotide, termed ZnSab, as a competitor (Fig. 7A). Using as a probe the same *csf1r* "X box" oligonucleotide, both unlabelled self oligonucleotide and the ZnSab oligonucleotide competed with the *csf1r* DNA-binding complexes seen in Fig. 5, while, as before, excess mutant oligonucleotides failed to compete. In the reverse experiment (Fig. 7B), proteins binding to labeled ZnSab oligonucleotide were cross-competed by unlabelled *csf1r* X box oligonucleotide as well as by unlabelled ZnSab. These experiments suggest that the same complexes bind to the *csf1r* promoter and to ZnSab. The ZnSab sequence, 5'-CATCTAAAGTGGGGAGAAA-3', cannot obviously be aligned with the mouse and human *csf1r* sequences, but the finding that this oligonucleotide competes for binding to those sequences suggests that binding is mediated through the C-terminal zinc fingers that are shared by EWS and FUS/TLS and that for FUS/TLS has been demonstrated to be the DNA binding domain (27) in the case of the ZnSab sequence.

Functional significance of binding to the TSS-flanking element

Both EWS and FUS/TLS mRNAs are expressed at high levels in murine BMM compared with other tissues based upon Affymetrix cDNA microarray profiling (symatlas.gnf.org), a finding that we have confirmed by quantitative real-time PCR analysis (not

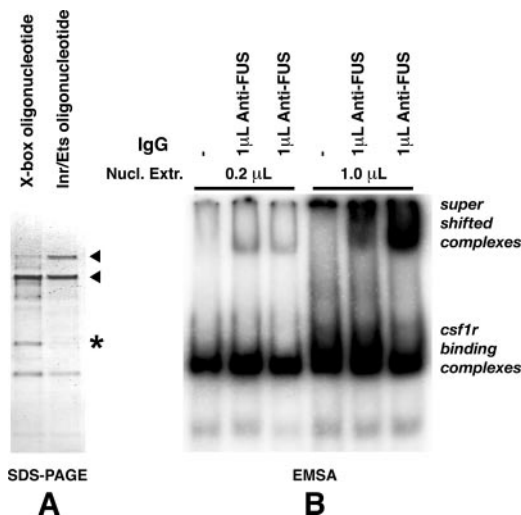


FIGURE 6. A, The 12% SDS-PAGE separation of proteins binding to double-stranded overlapping murine *csf1r* promoter oligonucleotides, stained with Coomassie blue. The "X box" oligonucleotide included the adjacent 5' PU.1 site and had the sequence 5'-GGGGAAGAGGAGCCA GTGCAACAGACAGGAAC-3'. The "Inr/Ets" oligonucleotide started partway through the conserved CCAGTG motif but extended farther downstream and had the sequence 5'-AGTGCAACAGACAGGAACGTGTT CAT-3'. Of the arrowed bands, the upper was identified by mass spectrometry as EWS, the lower as FUS/TLS. The band marked by the asterisk was identified as PU.1, which is known to bind the *csf1r* promoter. B, Ab supershift EMSA using BMM nuclear extracts following identification of EWS and FUS/TLS as proteins that bind the *csf1r* oligonucleotide. The major (lower) band is the normal EMSA complex; the probe has been run off the gel to enable greater separation of the complexes. The experiment was conducted with either 0.2 or 1.0 μ g of nuclear extract. In comparison to the control lanes lacking Ab, addition of anti-EWS or anti-FUS/TLS Abs (1 μ l in every case) produced a supershifted band (arrowed) on EMSA. The intensity of the supershifted band increased as more nuclear extract was added.

Table III. EWS and FUS/TLS peptides identified by MS/MS

Band	Peptide <i>m/z</i> (ESI)	Charge	MS/MS Sequence Obtained	Assignment	Corresponding Protein Sequence
A	713.34	2+	PM* (L/I) N (L/I) YTDR	FUS/TLS	TGQPMINLYTDR
A	512.25	2+	AA (L/I) DWFDFGK	FUS/TLS	AAIDWDFGK
B	603.27	2+	PGP (L/I) M*EQM*GG	EWS	PGPLMEQMGG
B	512.25	2+	AAVEWDFGK	EWS	AAVEWDFGK
B	833.02	3+	YGQESGGFSGPGENR	EWS	QDHPSSMGVYQGESGGFSGPGENR

*, methionine sulfoxide.

shown) and by the presence of sufficient protein to be isolated for mass spectrometric identification (Fig. 6A) and EMSA assays. FUS/TLS has been knocked out in the mouse germline, and the mutation is neonatal lethal (28). A defect in B lymphocyte development was observed, but pre-B cells from knockouts differentiated normally after transplantation into a wild-type background. The authors claim there was no myeloid phenotype (28), but the data actually show that the number of monocytes in peripheral blood was substantially reduced. In fact, the FUS/TLS mutant phenotype is not dissimilar to the selective monocyte and B lymphocyte depletion that is observed in *csflr*^{-/-} mice (29). In contrast, the EWS knockout (30) displays a cell-autonomous loss of B lymphocytes, but like the FUS/TLS knockout, has defects in meiosis. Although both FUS/TLS and EWS can specifically bind the *csflr* proximal promoter, we were interested to identify whether these factors were present at the *csflr* locus in vivo in unstimulated primary cells (BMMs). We conducted chromatin immunoprecipitation experiments for EWS, FUS/TLS, as well as for PU.1. Fig. 8 demonstrates the presence of EWS and (as expected) PU.1 but not FUS/TLS at the *csflr* locus in BMMs. Given that FUS/TLS is expressed in BMM and is clearly capable of binding the promoter in a cell-free system, this interesting result suggests that some mechanism intrinsic to the promoter architecture prevents the binding of FUS/TLS to the proximal promoter, at least in unstimulated BMMs.

Both FUS/TLS and EWS have multiple interaction partners and roles in basal transcription and RNA processing. Furthermore,

both have powerful activation domains, and EWS at least can act as a transcription factor in some circumstances (31). Using transfection analysis, we examined the possibility that by overexpression of these proteins in both macrophage and nonmacrophage cell types we could demonstrate them acting as transcription factors or repressors on the *csflr* gene. We transfected NIH3T3 cells with a 0.3-kb *csflr*-luciferase reporter plasmid simultaneously with either full-length EWS or full-length FUS/TLS expression vectors. In these cells, we found modest effects of overexpression. EWS repressed *csflr* reporter activity about half, whereas FUS/TLS produced a 2-fold activation (Fig. 9). Coexpressing PU.1 did not affect the pattern of results. In contrast, in RAW264.7 macrophage cells with various full-length or truncated forms of EWS or FUS/TLS, we observed no significant effect on the activity of a cotransfected *csflr* proximal promoter construct (data not shown). Although this suggests that EWS and FUS/TLS are incapable of acting as transcription factors in this context, we cannot rule out the possibility that the promoter architecture is already established by endogenous proteins so that overexpression contributes no additional effect. Furthermore, because RAW264.7 is a virally transformed cell line, it is possible that the results may reflect the transformed state of the cell, rendering it a poor model for primary macrophages in this instance.

We turned next to mutations of the promoter region to look at the role of each element. Mutation of the core CAGGAA (region

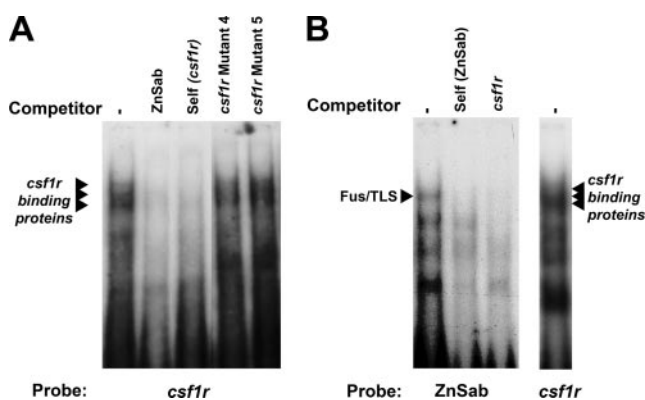


FIGURE 7. The *csflr* promoter DNA binding complexes are competed by the known FUS/TLS binding sequence ZnSab, and complexes observed on the ZnSab oligonucleotide are cross-competed by the *csflr* sequence. *A*, EMSA using BMM nuclear extract and labeled murine “X box” *csflr* probe. The *csflr*-binding complexes identified in Fig. 5 and that fail to be competed by a 100× molar excess of mutants 4 and 5 are effectively competed by a 100× molar excess of ZnSab, which contains a known FUS/TLS binding site. *B*, EMSA using BMM nuclear extract and labeled ZnSab probe. A ZnSab binding complex (presumably FUS/TLS) running in approximately the same location as the *csflr*-binding complexes is effectively competed both by unlabelled ZnSab and by a 100× molar excess of unlabelled murine X box *csflr* oligonucleotide.

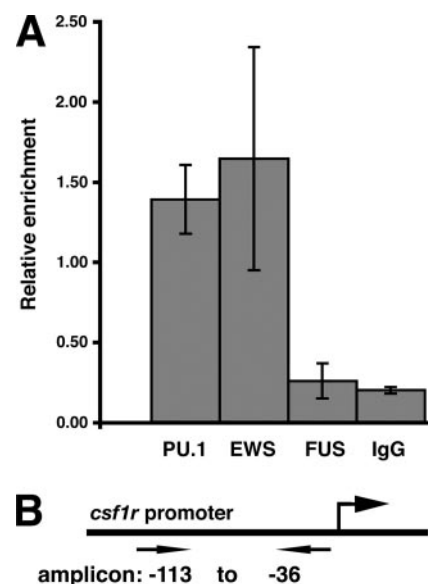


FIGURE 8. Chromatin immunoprecipitation indicates that EWS, but not FUS/TLS, is present at the *csflr* promoter. *A*, Immunoprecipitation from BMM chromatin of both EWS and of the known *csflr*-binding protein PU.1 reveals coprecipitation of the *csflr* promoter. Immunoprecipitations from the same extracts using anti-FUS/TLS Abs, or control IgG, fail to precipitate the *csflr* promoter. *B*, The amplicon used for chromatin immunoprecipitation experiments.

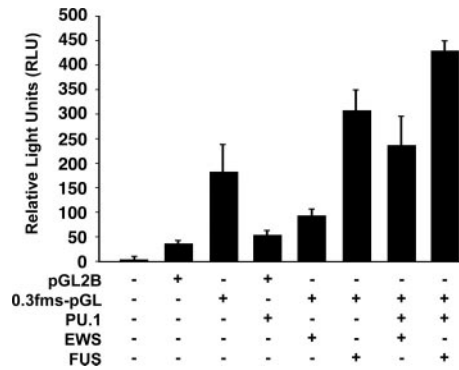


FIGURE 9. Transient transfection analysis in NIH3T3 (fibroblast) cells of the effect of full length wild-type EWS or FUS/TLS expression constructs on a 0.3-kb *csf1r*-luciferase reporter construct. Luciferase activity (\pm SEM) is shown for duplicate transfections. The micrograms of either pGL2-Basic (control) or 0.3-kb *csf1r*-luciferase (test) reporter plasmid was transfected along with 2 μ g of expression plasmid. Empty pEF6 vector was used as a control where no expression plasmid was used. Transfected cells were cultured for 24 h before being harvested and assayed for luciferase activity. A PU.1 expression vector was included to test for PU.1-dependent effects of EWS and/or FUS/TLS.

3) motif, which occurs at the very 3' end of the putative EWS and FUS/TLS site, ablates promoter activity in RAW264 macrophages (12), a finding that was reconfirmed here (data not shown). To further delineate the function of the FUS/TLS/EWS element, we made a number of other substitution mutations in the 0.5-kb *csf1r* promoter-luciferase reporter constructs. These mutant reporter plasmids were stably transfected into RAW264 cells. To avoid the inherent variation in single clones, pools of stable transfectants were assayed for luciferase activity. As shown in Fig. 10, mutation

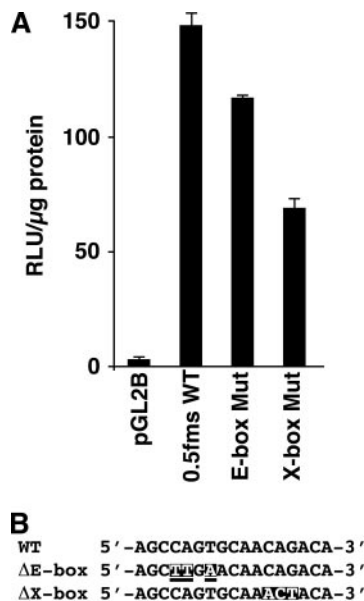


FIGURE 10. A, Stable transfection analysis of wild-type or mutated 0.5-kb *csf1r*-luciferase reporter constructs in the RAW264.7 macrophage cell line. Ten micrograms of reporter plasmid was transfected in the presence of 2 μ g pMC1Neo resistance plasmid, and pools of stable transfectants were selected for 7 days with G418 as reported in *Methods and Materials*. Equivalent cell numbers of the pooled stables were then plated overnight and assayed for luciferase activity. The results shown are the means of two independent experiments performed in triplicate, with the range of the data varying by $<10\%$ from the means. B, Mutations in the promoter region of the transfected luciferase reporter constructs.

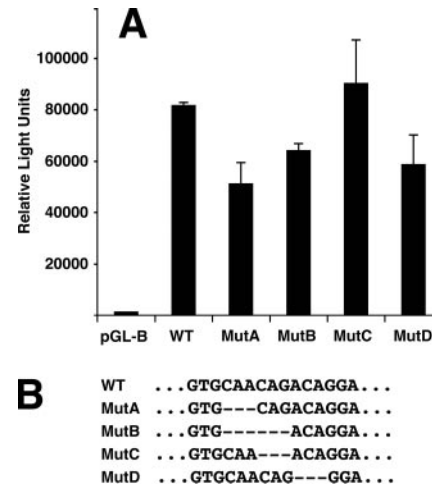


FIGURE 11. A, Transient transfection analysis in RAW264.7 (macrophage) cells of deletion mutations of the putative X box region immediately upstream of the transcription start sites of the *csf1r* promoter. Luciferase activity (\pm SEM) is shown for duplicate transfections. Reporter plasmids (10 μ g) comprising either wild-type or mutant promoters were transfected into RAW264.7 and the cells harvested at 24 h before being assayed for luciferase activity. B, Deletions in the promoter region of the transfected luciferase reporter constructs.

of the E box-like motif, CCAGTG, to CTTGTA caused a minimal reduction in promoter activity in stable transfectants. Mutation of the central triplet of the core EWS and FUS/TLS binding site (CAACAGACA) to CAAACTACA, which greatly reduced binding in the cold competition assays, caused $\sim 50\%$ reduction of promoter activity. A series of deletions in the CAACAGACA region (Fig. 11) also caused modest reductions in promoter activity in transient transfection assays. Given the fact that no single point mutation abolishes binding, the size of the motif, the potential for tethering these proteins to PU.1, and the fact that the zinc finger of these proteins is only likely to contact 3–4 bp, it is unlikely that any of these mutations completely abolishes EWS binding to the transfected promoter. Taken together, the data support the view that this element normally forms part of the *csf1r* proximal promoter complex.

Discussion

We have shown that the RNA binding zinc finger proteins EWS and FUS/TLS bind to positionally equivalent elements of the mouse and human *csf1r* promoters, located immediately upstream of the major transcription start sites, and that in vivo, EWS at least is present on the *csf1r* promoter. The extensive cold-competition studies indicate that binding is sequence-specific. It is formally possible that the band seen in our EMSA assays consists not of EWS and FUS alone, but of a complex of EWS or FUS with another protein that is actually responsible for the DNA-binding activity. We regard this as unlikely because the behavior of the EMSA complexes on the ZnSab probe (which is known to bind monomeric FUS/TLS directly) is similar to those on the *csf1r* probes, and because no other protein was seen in stoichiometric amounts on SDS-PAGE of the DNA binding complexes isolated specifically on the *csf1r* oligonucleotides (Fig. 6). We confirmed the earlier observation that, in vitro, FUS/TLS binds to the consensus binding sites for the myeloid zinc finger protein, Mzf1, and we extended the finding to EWS. This observation suggests that the binding is mediated by the zinc fingers, as previously inferred (27). Given the size of the binding site (11 bp) compared with the 4–5 bp required for Mzf1 binding (see www.jaspar.com), and the

fact that no single base pair change abolishes binding, there may be multiple binding sites for the two factors in the *csflr* proximal region. ChIP experiments show that in vivo, EWS but not FUS/TLS is present at this locus, a surprising result given that the known interactions with PU.1 would be expected to preferentially stabilize FUS/TLS binding. Although an interaction between EWS and PU.1 is not impossible, it has not been reported to date. It may be that PU.1 has another partner in vivo that actually excludes FUS/TLS binding in resting (unstimulated cells) and that in some other situations FUS/TLS binding is enabled. Alternatively, the FUS/TLS binding site may be obscured in vivo in some other way. Naturally, until the *csflr* locus is examined by ChIP under a wider range of conditions, it is impossible to formally exclude a role for FUS/TLS as well as EWS at this promoter.

Part of the reason that interactions of PU.1 with FUS/TLS are known is that FUS is commonly associated with translocations in myeloid leukemias (32). In general, tumor-promoting fusions of both FUS and EWS involve the association of the N-terminal *trans*-activation domains with DNA-binding domains of classical transcription factors, notably members of the Ets family. Such fusions might bring together in a single molecule activities that are normally contributed separately to myeloid promoters. Alternatively, the fusion proteins might interfere with optimal transcription or processing of genes such as the *csflr*, which are required for terminal myeloid differentiation. If Mzf1, FUS/TLS, and EWS bind to similar DNA sequences, there is the formal possibility that like Pax5, Mzf1 (and possibly related C2H2 zinc finger proteins such as PML and the Gli1 family (33, 34)) would act as a repressor of myeloid-specific transcription by competing directly for binding to this important proximal promoter element. Mzf1 is expressed in pluripotent stem cells and early myeloid progenitors, but it is absent from mature myeloid cells. The decline in Mzf1 with myeloid lineage commitment could permit activation of promoters like that of the *csflr*. The knockout of Mzf1 in mice does, indeed, lead to a myeloid hyperproliferative syndrome (35). In a similar vein, Tagoh et al. (36) recently showed that Pax5, which represses *csflr* expression in B cells, does so by directly binding to the AGTG CAACAGACAGGAACGTG element of the *csflr* promoter, immediately displacing RNA polymerase II while still permitting PU.1 binding. Although these authors suggested that Pax5 would disrupt the interaction between PU.1 and the transcription initiation complex, our results suggest that it could also act by abrogating binding of EWS to this site.

Both EWS and FUS/TLS are TATA-associated factors (there is a third family member, TAF15, which was not detected in this study). Each of these factors has a powerful N-terminal transactivation domain that can bind to the Zfm1 (or Sf1) protein. There is evidence that the proteins are associated with separate pools of TFIID and with the RNA polymerase II holoenzyme (37). Thus, given this ability, and the location of the binding sites on the promoters, we propose that EWS essentially substitutes for TATA-binding protein and serves a similar function to Sp1 on GC-rich TATA-less promoters (38). In fact, this role of EWS (and/or FUS/TLS) could be a more general feature even of the CpG-rich class of TATA-less promoters. The systematic analysis of start sites by CAGE in the FANTOM3 project revealed substantive G anisotropy (i.e., enrichment of Gs on the upper strand) within CpG island promoters, which is the major promoter class in the mammalian genome (6).

It is clear that different promoters use different sets of basal transcriptional factors in addition to the general transcription factors (7). For example, TBP is not essential for TATA-less promoters (39). In contrast, TIC-2 and TIC-3 (incompletely characterized TATA/Inr cofactors) are necessary for the in vitro reconstitution of

transcription from TATA-less promoters (40). These factors have yet to be identified but clearly TLS/FUS and EWS are candidates for these or similar activities. Interestingly, Martinez et al. (40) who described TIC-2 noted that one component of the fraction is TAF15 (TFII68), which is the third member of the EWS family.

EWS and FUS/TLS are remarkably multifunctional proteins, with clear examples of specific roles in transcription, splicing, and RNA transport. To date, FUS/TLS has been ascribed the greatest diversity of roles. Despite the close similarity between the two molecules, clear structural and functional differences exist. For example, FUS/TLS (but apparently not EWS) is associated with trafficking RNA to dendritic spines (41–43) or focal adhesions (44), while most specific interactions with splicing or transcription factors have been described for either FUS/TLS or EWS but not for both.

The final question arising from our data is therefore whether the functions of the two proteins on myeloid promoters can be redundant, especially because the EWS knockout does not lead to a depletion in the myeloid cell population (30). Because of the relatively loose binding specificity and propensity for poly(G) binding, protein-protein interactions are likely to play a role in the specificity of association of EWS and FUS/TLS with the *csflr* promoter. FUS/TLS has been shown to bind and regulate functions of PU.1, including splicing (45, 46), so there is a clear possibility of functional interactions between the two proteins on myeloid promoters, especially because on the *csflr* promoter it is FUS/TLS that appears to bind immediately downstream of the PU.1 site. In contrast EWS, but not FUS/TLS, contains an RNA polymerase II-like domain. In the case of the EWS-WT1 fusion, this region is capable of being phosphorylated by Abl kinases, leading to the initiation of paused transcriptional complexes (47). Whether this occurs with native EWS is not known.

Although we favor a role for EWS in transcriptional initiation, it could alternatively contribute to splicing and/or transcriptional elongation. It might also participate in the phenomenon of exon tethering (48) through its dual RNA- and DNA-binding abilities. The clear evidence of specific binding to the TSS provides the basis and impetus for future mechanistic studies.

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Disclosures

The authors have no financial conflicts of interest.

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