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MIP1α Nuclear Protein (MNP), a Novel Transcription Factor Expressed in Hematopoietic Cells That Is Crucial for Transcription of the Human *MIP-1α* Gene

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Murine macrophage inflammatory protein 1 alpha (MIP-1α) and its human equivalent (GOS19, LD78, or AT464) are members of the -C-C- family of low-molecular-weight chemokines. Secreted from activated T cells and macrophages, bone marrow-derived MIP-1α/GOS19 inhibits primitive hematopoietic stem cells and appears to be involved in the homeostatic control of stem cell proliferation. It also induces chemotaxis and inflammatory responses in mature cell types. Therefore, it is important to understand the mechanisms which control the expression of *MIP-1α*/*GOS19*. Previous work has shown that in Jurkat T cells, a set of widely expressed transcription factors (the ICK-1 family) affect the *GOS19* promoter. One member, ICK-1A, behaves as a strong negative regulator. In this communication, we provide evidence that the pathway of induction in the macrophage cell line U937 is different from that in Jurkat cells. Furthermore, we show that the ICK-1 binding site does not confer negative regulation in U937 cells. We provide evidence for an additional binding site, the *MIP-1α* nuclear protein (MNP) site, which overlaps the ICK-1 site. Interaction of nuclear extracts from various cell lines and tissue with the MNP site leads to the formation of fast-migrating protein-DNA complexes with similar but distinct electrophoretic mobilities. A mutation of the MNP site which does not abrogate ICK-1 binding inactivates the *GOS19.1* promoter in U937 cells and reduces its activity by fourfold in Jurkat cells. We propose that the MNP protein(s) binding at the MNP site constitutes a novel transcription factor(s) expressed in hematopoietic cells.

Murine macrophage inflammatory protein 1 alpha (MIP-1α [48]) and its human equivalent (GOS19 [2, 12, 44], LD78 [34, 37], or AT464 [20, 53]) are members of the -C-C- family of low-molecular-weight chemokines. This family is characterized by a similar gene organization (three exons and two introns) and similar protein structures (5, 22, 30, 32, 38, 48). In particular, each member has four conserved cysteine residues located in almost identical locations in the primary amino acid sequence. In keeping with other members of the family, MIP-1α/GOS19 has proinflammatory properties and has been shown to activate mast cells and basophils, to be chemotactic for T cells and monocytes, and to induce an oxidative burst in neutrophils (1, 10, 21, 39, 46). In addition to these inflammatory properties, MIP-1α/GOS19 has been shown to inhibit cell cycle progression of hematopoietic stem cells (3, 4, 8, 9, 13, 14, 17, 43), and it may be identical to the in vivo bone marrow stem cell inhibitor (SCI) described by Lord and his colleagues (25). SCI is secreted by macrophages present in normal murine bone marrow (24, 26). In regenerating bone marrow, little MIP-1α/GOS19 is secreted, but macrophages secrete a stem cell stimulator (SCS) (23). Therefore, it is important to understand the switch in expression from SCI to SCS during homeostatic regulation of stem cell proliferation and to identify the mechanisms controlling the expression of *MIP-1α*/*GOS19*.

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Although the murine genome contains only one gene for MIP-1α, there are three known human equivalent genes located on chromosome 17, bands q11 to q21. Two encode functional copies of the MIP-1α coding sequence (*GOS19.1* and *GOS19.2*), and the third encodes a nonfunctional pseudogene which lacks the 5′ flanking region and most of the first intron (*GOS19.3*) (2, 16, 20, 34). Individuals have only one copy of the *GOS19.1* gene, while the *GOS19.2* and *GOS19.3* genes are variable in their copy number. *GOS19.1* and *GOS19.2* show 94% nucleotide sequence homology over 4 kb. *GOS19.2*, however, has a repetitive *Alu* sequence inserted 294 bp upstream of the transcription initiation site. It has been shown that this *Alu* sequence has little or no regulatory role in transcription (36). Both have been shown by the reverse-transcriptase PCR method to be actively transcribed (34), and although the secreted forms show three amino acid differences, no dissimilarity in biological activities has been shown to date (21, 46).

Under nondisease situations, *GOS19* is expressed and secreted by activated T cells, activated monocytes, and dendritic cells (29, 47, 52, 54). Elevated levels of *GOS19* expression and secretion in transformed B cells and in fibroblast-like cells in chronic inflammation induced by *Schistosoma mansoni* infection have also been reported (27, 42). The cell type expression and pathways of induction of *GOS19* in monocytes and T cells have been well characterized (15, 28). Prior to cellular activation, no *GOS19* RNA is detectable. However, treatment of U937 cells with phorbol 12-myristate 13-acetate (PMA) induces high levels of *GOS19* mRNA transcripts which can be detected within 2 h (34). Treatment of Jurkat T cells with PMA plus phytohemagglutinin (PHA) also results in cellular activation and expression of *GOS19* mRNA (19, 37, 54). Further-

more, de novo protein synthesis is required for expression of *GOS19* in Jurkat cells but is not required in U937 cells (12). These reports suggest a basic difference in the mechanisms of transcriptional activation between these two cell lines.

Functional domains of the promoter region of *GOS19.1* and *GOS19.2* in the Jurkat cell line and the erythroleukemic cell line K562 have been studied elsewhere (36). A small domain of 22 bp approximately 100 bp upstream of the transcription start site which acts as a suppressor of transcription in nonactivated Jurkat cells was defined. Interestingly, this domain is also the binding site of positive regulatory elements and is also required for full expression upon activation of Jurkat cells. This domain contains a 13-bp sequence motif, termed the ICK-1 element, which binds four identified nuclear factors (ICK-1A, -B, -C, and -D). At least one of these, ICK-1A, is required for suppression of transcription in nonactivated Jurkat cells. Just upstream from the ICK-1 element, there is a consensus binding site for another transcription factor, variously termed CLE0 or CK2, which is present on several hematopoietic cytokine gene promoters (33, 35, 41). However, deletion of this sequence has been shown to have no effect on the activity of the *GOS19.1*/*GOS19.2* promoter in Jurkat cells in transient-transfection assays (36).

In this communication, we show that in contrast to Jurkat cells, the region containing the ICK-1 domain does not act as a transcriptional suppressor in nonactivated U937 cells, although it is required for full expression following cellular activation with PMA and PHA. We have identified an additional *cis*-acting sequence overlapping the ICK-1 target site which binds a novel transcription factor(s), *MIP-1*α nuclear protein (MNP). In essence, interaction of the MNP factor(s) with its target site is crucial for transcriptional activity of the *GOS19.1* gene.

MATERIALS AND METHODS

Generation of recombinant plasmid clones. A 992-bp fragment (from bp −813 to +179, relative to the transcription start site) of the *GOS19.1* gene was cloned from U937 cell DNA by using PCR (Perkin Elmer-Cetus Gene Amp PCR System 9600). Primers were designed with *Bam*HI sites engineered on each 5′ end (upstream primer BU3 [5′-GCACCCGGGATCCGCCACTTCATTCATTT TAGAGA-3′] and downstream primer BU4 [5′-GCACCCGGGATCCCCCAC AACGAAACTCAGACTCA-3′]; Applied Biosystems 391 DNA synthesizer, PCR-MATE). The PCR product was cloned into the *Bam*HI site of plasmid pUC19 (Gibco-BRL, Gaithersburg, Md.) and transfected into *Escherichia coli* DH5α (Gibco-BRL). The DNA sequence was verified by dideoxynucleotide sequencing with Sequenase version 2.0 (U.S. Biochemicals, Cleveland, Ohio) according to the manufacturer's protocol. This plasmid, pGSP992, was then used for the further cloning of a set of 5′ deletion mutants for functional assays of promoter activity and for the cloning of fragments of the promoter for mobility shift assays.

To facilitate cloning of the 5′ deletion mutants, pGSP992 was digested with *Bam*HI (5′ end of insert) and *Xho*I (+51). The resulting 864-bp fragment (−813 to +51) was isolated and cloned into the *Bam*HI-*Xho*I site of pBluescript II KS$^+$ (Stratagene, La Jolla, Calif.) and was called plasmid pGSP864. pGSP864 was digested with *Xho*I and one of the following enzymes, with cleavage sites located at the indicated positions relative to the transcription start site: *Sma*I (−813), *Asp*700i (−645), *Pvu*II (−368), *Hin*cII (−265), or *Eco*RV (−152). The resulting fragments were directionally cloned into the *Sma*I-*Xho*I site of the pGL2-Basic vector (Promega, Madison, Wis.) upstream of the luciferase reporter gene such that transcription would begin at the *GOS19.1* start site and the first ATG encountered would be that of the luciferase gene. pGSP846 was also cut with *Cfo*I (−532), treated with T4 DNA polymerase (New England BioLabs, Beverly, Mass.), and cut with *Xho*I. The resulting fragment was also cloned into the *Sma*I-*Xho*I site of pGL2-Basic. These reporter plasmids were designated as follows: pGSP-813luc, pGSP-645luc, pGSP-368luc, pGSP-265luc, pGSP-152luc, and pGSP-532luc. pGSP-119luc, pGSP-87luc, pGSP-76luc, and pGSP-45luc were constructed by using PCR with appropriate upstream primers (5′-GTTGTG CAACTTAGCATGACAGCATCACTA-3′, 5′-TTAAAAATTTCCCTCCTCA CC-3′, 5′-CTCCTCACCCCCAGATTCCATTTCC-3′, and 5′-CGCCAGGGCT GCCTAT-3′) and a downstream primer past the *Xho*I site of pGSP992 (5′-TG AGCAGGTGACGGAATG-3′). The resulting PCR products were cut with *Xho*I and cloned into the *Sma*I-*Xho*I site of pGL2-Basic. Since PCR was used in the generation of these plasmids, all were sequenced for verification as previously described.

To prepare DNA fragments for electrophoretic mobility shift assays (EMSA), pGSP992 was cut with the following group of restriction enzymes located at the indicated positions relative to the transcription start site of *GOS19.1*: *Bam*HI (−813)-*Asp*700i (−645), *Asp*700i-*Cfo*I (−532), *Cfo*I-*Pvu*II (−368), *Pvu*II-*Hin*cII (−265), *Hin*cII-*Eco*RV (−152), or *Eco*RV-*Alu*I (−18). pGSP992 was also cut with *Alu*I alone to enable isolation of a 196-bp fragment from −17 to +179. The digests which included *Cfo*I were treated with T4 DNA polymerase to create blunt ends. The *GOS19.1* promoter fragments were separated by electrophoresis on 2% low-melting-point agarose or 10% polyacrylamide and eluted. *Bam*HI linkers (Gibco-BRL) were ligated on each end with T4 DNA ligase (New England BioLabs), cut with *Bam*HI, ligated into the *Bam*HI site of the pUC19 plasmid, and transformed into *E. coli* DH5α. The resulting plasmids were designated as follows: pGSP-813/-646, pGSP-645/-533, pGSP-532/-369, pGSP-368/-266, pGSP-265/-153, pGSP-152/-18, and pGSP-17/+179.

Cell culture and transfections. The human histiocytic lymphoma cell line U937 (45), the human fibroblast cell line HeLa S3, the human monocytic cell line THP-1, the mouse embryonal carcinoma cell line F9, and the human chronic myelogenous leukemic cell line K-562 were obtained from the American Type Culture Collection (Rockville, Md.). The human leukemic cell line Jurkat was obtained from The Ohio State University cell line depository. Raji, an Epstein-Barr virus-positive (EBV$^+$) Burkitt's lymphoma B-cell line, and BJAB, an EBV$^-$ B-cell line, are regularly cultured in our laboratory. All cell lines were maintained in RPMI 1640 medium (Gibco-BRL) supplemented with 10% fetal bovine serum (Intergen Company, Purchase, N.Y.). Cells were grown logarithmically to a density of 8×10^5 cells per ml.

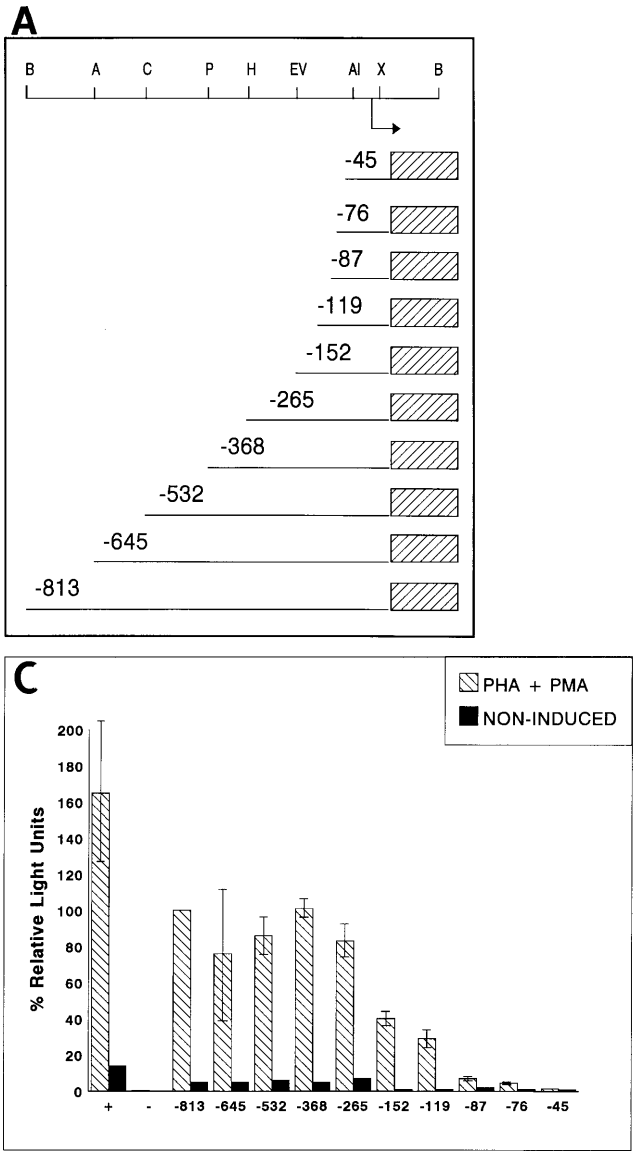
For transfection of U937 and Jurkat cells, pools of 36×10^6 cells were washed twice with OptiMEM reduced-serum medium (Gibco-BRL) and split into 12 equal parts, each part containing 3×10^6 cells in 800 μl. These were seeded into 35-mm cell culture wells. For U937 cells, each well was transfected with 0.75 pmol of one of the *GOS19.1* promoter deletion-luciferase plasmids by using 15 μl of Lipofectin reagent (Gibco-BRL) according to the manufacturer's protocol. As a negative control, one set of cells was transfected with pGL2-Basic (promoterless luciferase plasmid; Promega). As a positive control, another set of cells was transfected with pGL2-Control (Promega), in which the luciferase gene is under the control of the simian virus 40 early promoter. Initially, transfection efficiencies were normalized by cotransfection with 1 μg of pCH110 (Clontech, Palo Alto, Calif.), a simian virus 40 early promoter–β-galactosidase plasmid. The cells were gently rocked and incubated for 9 h at 37°C. Four milliliters of RPMI 1640 supplemented with 20% fetal bovine serum was added to each well, and the cells were incubated for an additional 32 h. At that time, each was split, one-half was treated with PMA (Sigma, St. Louis, Mo.) at a final concentration of 20 ng/ml and PHA (Sigma) at a final concentration of 1 μg/ml, and the other half was left untreated. The cells were incubated with gentle rocking for an additional 8 h and harvested. Transfections were repeated with two different DNA preparations three times.

Jurkat cells were transfected and treated in the same manner, with the following changes. Twelve microliters of LipofectAMINE (Gibco-BRL) was used according to the manufacturer's protocol. Initial incubation before the addition of medium was for 5 h. Cells were then incubated for 7 h, treated with PHA and PMA, and incubated for an additional 8 h before harvesting.

Luciferase assay. Harvested cells were lysed by using the Promega luciferase assay system according to the manufacturer's protocol. The lysate was centrifuged, and 10 μl of the supernatant was assayed for luciferase activity, which was measured in light units with a Berthold (Nashua, N.H.) Lumat 9501 luminometer with a 100-μl injector (6, 40, 51). Light units after normalizing for total protein were expressed as a percentage of the activity of the full-length wild-type (wt) promoter (pGSP-813luc).

Nuclear extracts and EMSA. Nuclear extracts were prepared from various cell lines and brain tissue, essentially according to the method described by Dignam et al. (7). For U937 nuclear extracts only, the following modifications were made. The low-salt buffer contained 0.1 M KCl, and the high-salt buffer contained 1 M KCl, thus preventing a sudden high change in salt concentration and lysis of the nuclei. All extracts were dialyzed against 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9) at 4°C, 20% glycerol, 100 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol for 4 h at 4°C, frozen in aliquots in an ethanol-dry ice bath, and stored at −80°C. U1240, U1242 (two glioblastoma cell lines), NIH 3T3, and human brain nuclear extracts were a kind gift from Subir Ray and Maqsood Chotani (The Ohio State University, Columbus). An SP1 consensus sequence oligonucleotide (Promega) was used to confirm the activity of nuclear proteins in all nuclear extracts.

For EMSA analysis, appropriate plasmids were digested with *Bam*HI and the fragments were isolated. After dephosphorylation with shrimp alkaline phosphatase (U.S. Biochemicals), the fragments were end labeled with [γ-^{32}P]ATP (DuPont NEN, Boston, Mass.) by using T4 polynucleotide kinase (New England BioLabs). Binding reactions were performed with a mixture containing 25 mM HEPES (pH 7.5), 10% glycerol, 50 mM KCl, 1.5 mM dithiothreitol, and 0.1 mM EDTA for 20 min at room temperature. In a 20-μl reaction mixture, 5 μg of poly(dI-dC) and 15 μg of crude nuclear extract were used. The mixture was kept on ice until the addition of 25,000 cpm (approximately 1 ng unless otherwise noted) of probe. In competition experiments, unlabeled competitor was added



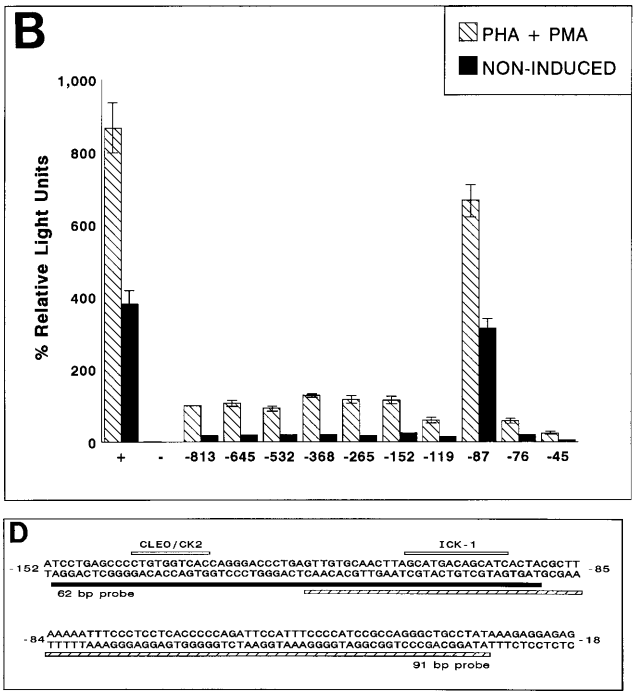


FIG. 1. Transient expression of *GOS19.1* promoter-luciferase constructs. (A) Schematic of the 992-bp fragment of the *GOS19.1* gene cloned from U937 cells, with appropriate restriction sites indicated. B, *Bam*HI; A, *Asp*700i; C, *Cfo*I; P, *Pvu*II; H, *Hin*cII; EV, *Eco*RV; Al, *Alu*I; and X, *Xho*I. Arrow, transcription start site; striped boxes, luciferase reporter gene. (B and C) Successive 5′ promoter deletions (indicated by numbers relative to the transcription start site) were linked to the luciferase reporter in the pGL2-Basic vector, and each construct was individually transfected into Jurkat cells or into U937 cells. Light units after normalization for total protein were expressed as a percentage of the activity of the full-length (pGSP-813luc) wt promoter. The graphs of Jurkat cells (B) and U937 cells (C) are the averages of three separate experiments, with standard deviations indicated. +, simian virus 40 early promoter-luciferase construct; −, promoterless construct; black columns, results from nonstimulated cells; striped columns, results after stimulation with PHA and PMA for 8 h. (D) Nucleotide sequence of the *GOS19.1* promoter region from −152 to −18. The positions of the overlapping 62 (heavy black line)- and 91 (striped line)-bp probes subsequently used in EMSA and the identified CLE0/CK2 binding site and ICK-1 element are indicated.

immediately before the probe. After incubation, the samples were electrophoresed on either 5 or 6% polyacrylamide (acrylamide-bisacrylamide [29:1]), and the gel was dried and visualized by autoradiography.

Methylation interference analysis. Methylation interference analysis was performed as previously described (50). 5′-End-labeled single-stranded synthetic oligonucleotides were annealed to cold complementary oligonucleotides and were methylated with dimethyl sulfate (Aldrich Chemical Company, Inc., Milwaukee, Wis.). The binding reaction was performed as described above for EMSA with 200,000 cpm of end-labeled probe, 8 μg of dI-dC, and 60 μg of crude nuclear extract. The complexes and free probe were separated on 5% polyacrylamide, the appropriate bands were cut out, and the DNA was electroeluted. After precipitation, the DNA from each band was cleaved with piperidine (Sigma). The cleaved upper strand was electrophoresed on a 12% polyacrylamide (acrylamide-bisacrylamide [19:1]) sequencing gel, and the lower strand was electrophoresed on a 20% gel. The resulting G tracts were visualized by autoradiography.

Mutation construction and insertion into the full-length promoter. Two 62-bp oligonucleotide fragments were synthesized as previously described. Each contained transvertional mutations in only the four upstream contact points of the MNP-1 complex as identified by methylation interference (62M1U [5′-TCCT GAGCCCCTGTGGTCACCAGGGACCCTGAGTTTTTAAAATTAGCA TGACAGCATCACTA-3′] and 62M1L [5′-TAGTGATGCTGTCATGCTAA TTTTAAAAACTCAGGGTCCCTGGTGACCACAGGGGCTCAGGA-3′]). For EMSA analysis, one of these oligonucleotides was end labeled and annealed to the cold complementary strand as previously described.

The oligonucleotide 62M1U was used as the upstream primer in conjunction with BU4 as the downstream primer in a PCR with plasmid pGSP992 as the template. In a separate reaction, BU3 was used as the upstream primer in conjunction with 62M1L as the downstream primer, again with pGSP992 as the template. The two resulting PCR products (341 and 735 bp, respectively) were band isolated and phosphorylated with ATP (Sigma) as described previously. Then, 10-ng amounts of each of these PCR products were used together as the template in a PCR, in the absence of any primers. After five cycles of PCR, 400 ng of both BU3 and BU4 was added to the reaction mixture, and the PCR was continued for 25 more cycles. The resulting 1,018-bp product was cleaved with *Sma*I and *Xho*I, band isolated and directionally cloned into the *Sma*I-*Xho*I sites of the pGL2-Basic vector, and transfected into *E. coli* STBL2 (Gibco-BRL). This plasmid, pGSP-813M1luc, was sequenced as described for verification. pGSP-813M1luc was subsequently used in transfections and functional assays as described above.

RESULTS

Promoter activity in T cells and macrophages. Functional studies of the *GOS19.1* promoter were carried out by transient-transfection assays with Jurkat and U937 cells. Jurkat cells can be activated with PHA and PMA, and U937 cells can be induced to differentiate into macrophages with PMA treatment. The set of sequential 5′-terminal deletions shown in Fig. 1A were fused to a luciferase reporter gene and transiently transfected into Jurkat and U937 cells. Since it has been shown that

some cell lines transport luciferase out of the cell after a variable amount of time, both transfection and incubation times had to be predetermined for each cell line (31). After the predetermined incubation time, the transfected cultures were split into 2 equal parts. One half was treated with PHA and PMA, and the other half was left untreated. After another 8 h, the cultures were harvested, cellular extracts were prepared, and the promoter activity for each set of transfections was determined and expressed as percent light units compared with the full-length wt promoter pGSP-813luc (Fig. 1B and C). The simian virus 40 early promoter is known to be responsive to phorbol esters (49). As thus expected, pGL-2Control (the simian virus 40-positive control plasmid) had activity in both cell lines and was induced to a higher level of expression after cellular activation. The promoterless negative control plasmid, pGL-2Basic, showed only background activity in both cell lines, with or without activation.

In both Jurkat cells and U937 cells, there was very little activity from the full-length *GOS19.1* promoter (pGSP-813luc) in nonactivated cells, but a considerable induction of expression was obtained upon cellular activation. In nonactivated Jurkat cells, sequential deletion of the promoter to position −119 relative to the transcription start site had very little effect on activity. However, deletion of the sequence which contains the ICK-1 element (pGSP-87luc) between −119 and −87 (Fig. 1D) resulted in a substantial increase in the percentage of relative light units. These results further extend and confirm the previous findings of Nomiyama et al. (36) who, using 3′ deletion mutants, have shown that a negative regulatory factor, which is active in Jurkat cells, binds to the ICK-1 element.

In nonactivated U937 cells, sequential deletion of the promoter, including the region between positions −119 and −87, had little effect on the low level of promoter activity (Fig. 1C). Therefore, it appears that there is no suppressor activity associated with the region containing the ICK-1 element in these cells. In activated U937 cells, two regions appear to be important for full promoter activity. Deletion of the region between positions −265 and −152 (pGSP-152luc) and the region between −119 and −87 (pGSP-87luc) both caused a marked reduction in the percentage of relative light units. The region between these two domains (−152 to −119) contains the putative CLE0/CK2 binding site (Fig. 1D), which is apparently not required for full promoter activity in transient-expression assays. The focus of this communication is the domain located in the 32 bp between −119 and −87 containing the ICK-1 sequence, which is essential for transcription (Fig. 1D).

Analysis of DNA-binding proteins by EMSA. Extracts of nuclear proteins were prepared from Jurkat and U937 cells. The 135-bp fragment containing the ICK-1 element and the putative CLE0/CK2 site was excised from pGSP-152/-18 plasmid DNA, gel purified, end labeled, and used as a probe to detect sequence-specific DNA-binding proteins by EMSA analysis. Figure 2, lanes 1 to 5, shows the presence of several shifted labeled complexes using Jurkat (lane 2) and U937 (lane 4) nuclear extracts, which were shown to be specific by competion with an excess of unlabeled self DNA (lanes 3 and 5). (No difference was seen by using nuclear extracts from activated or nonactivated cells [data not shown].) Most importantly, fast-migrating complexes were obtained by using U937 and Jurkat nuclear extracts. These same bands were seen when extracted proteins were bound to a 91-bp fragment located between −119 and −29 (Fig. 2, lanes 6 to 10) and also to a 62-bp fragment located between −151 and −90 (Fig. 2, lanes 11 to 15). These results suggested that the faster-migrating complexes were due to the binding of nuclear proteins at a site defined by the overlap between the 62-bp fragment and the

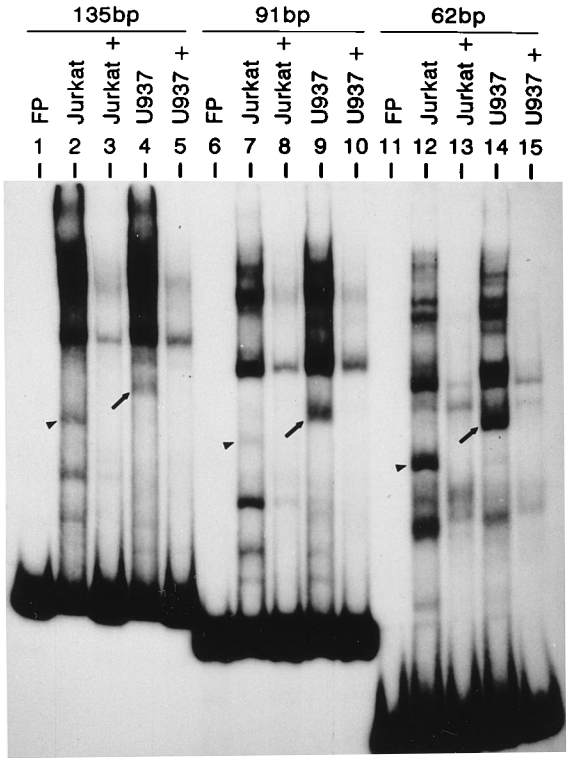


FIG. 2. EMSA of overlapping 135-bp (lanes 1 to 5), 91-bp (lanes 6 to 10), and 62-bp (lanes 11 to 15) fragments. Labeled probes (see Fig. 1D) were incubated with crude nuclear extracts as described in Materials and Methods. Lanes: 1, 6, and 11, free probe (FP); 2, 7, and 12, Jurkat cell nuclear extract; 3, 8, and 13, Jurkat cell nuclear extract plus 30× cold self-competitor; 4, 9, and 14, U937 cell nuclear extract; lanes 5, 10, and 15, U937 cell nuclear extract plus 30× cold self-competitor. The U937-specific MNP-1 complexes formed with each probe are indicated by arrows. The Jurkat cell-specific MNP-2 complexes formed with each probe are indicated by arrowheads. By using the 91-bp probe, the MNP-2 complex was faint, probably because the binding site is quite near the 5′ end of this sequence.

91-bp fragment, i.e., in a 30-bp sequence located between −119 and −90 containing the ICK-1 element. The faster-migrating Jurkat cell complex was faint with the 91-bp probe, which was subsequently found to be caused by the fact that the binding site for this factor is quite near the 5′ end of this probe. We synthesized a 30-bp double-stranded DNA fragment corresponding to this sequence and used it in EMSA. The fast-migrating complexes formed poorly with this fragment, and we assume that binding of the protein factor(s) to this site requires a larger stretch of DNA (data not shown). For this reason, all further experiments were carried out with the larger 62-bp DNA probe. As seen in Fig. 3, at least four complexes were identified which are due to binding of the ICK-1A, -B, and -C factors and the CLE0/CK2 factor (complexes 3, 4, 6, and 5, respectively). This was determined by competition with a 30-bp oligonucleotide containing the ICK-1 element (lane 5) and with a double-stranded oligonucleotide defining the CLE0/CK2 sequence from the granulocyte-macrophage colony-stimulating factor gene promoter (41) (lane 6). In our hands, nuclear extracts often fail to form the most slowly migrating ICK-1 complex (ICK-1D). Titration of cold competitor showed that the U937 and Jurkat unique fast-migrating complexes (complexes 1 and 2) were very sequence specific. As little as a 5-fold molar excess of cold fragment was sufficient to cause competition of these complexes with the labeled probe (data not shown), in contrast to ICK-1A, which required a 30- to

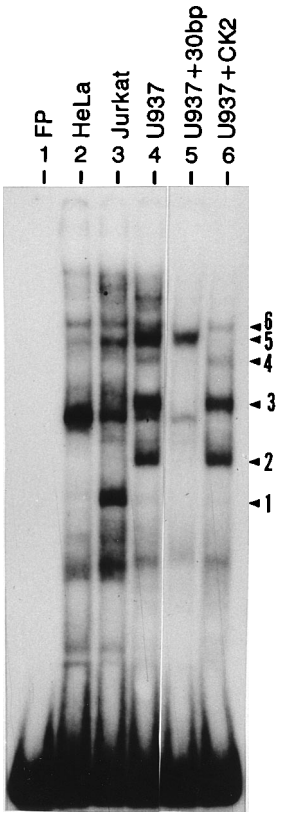


FIG. 3. EMSA of 62-bp fragment and competition identification of DNA-protein complexes. EMSA were performed as described in Materials and Methods. Complexes were inhibited with a 50-fold molar excess of oligonucleotide representing the 30-bp overlap region −119 to −90, which includes the ICK-1 element (lane 5) and an oligonucleotide containing the CLE0/CK2 site from the granulocyte-macrophage colony-stimulating factor promoter (lane 6). Identified complexes: MNP-2 (1), MNP-1 (2), ICK-1A (3), ICK-1B (4), CLE0/CK2 (5), and ICK-1C (6). FP, free probe.

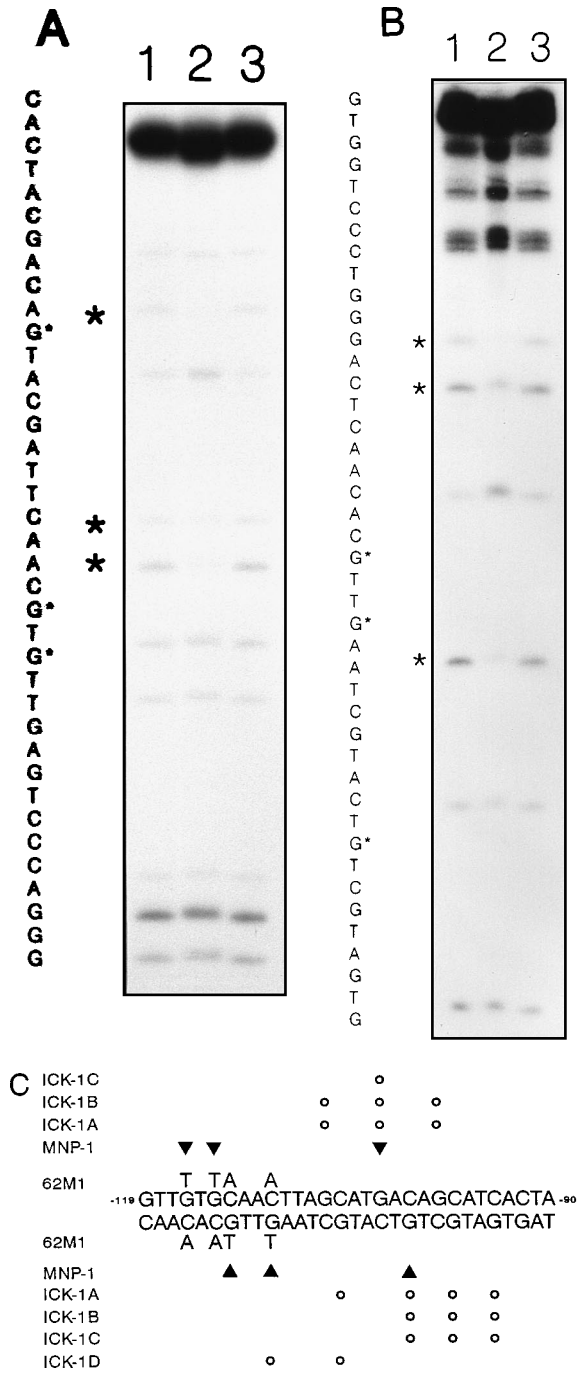


FIG. 4. Contact point analysis of MNP-1. Methylation interference analysis of the interaction of MNP-1 with the 62-bp fragment (−151 to −90) was carried out as described in Materials and Methods. (A) Contact point identification on the upper strand; (B) contact point identification on the lower strand. Lanes: 1, G tracts of methylated probes not used in EMSA; 2, G tracts of methylated probes complexed with MNP-1; 3, G tracts of free methylated probes from EMSA. Asterisks, protected Gs. Nucleotide sequences of each labeled strand are indicated to the left, with protected Gs indicated. (C) Comparison of protected Gs of the ICK-1 complexes (○) and the MNP-1 complex (▼). Base changes which constitute the 62M1 mutation are also indicated.

50-fold molar excess of competitor to achieve the same level of competition. Nomiyama et al. (36) have shown that ICK-1A has a low binding affinity for the ICK-1 element. Consensus binding site double-stranded oligonucleotides for 12 known transcription factors, SP1, NF1/CTF, AP1, AP2, AP3, NF-κB, GRE, CREB, OCT-1, PU.1, SRE, and CLE0/CK2 (11, 18), were used as cold competitors at a 100-fold molar excess. None could compete with the probe for binding of the faster complexes (data not shown). Therefore, we propose to call these complexes MNP-1 (U937 cells) and MNP-2 (Jurkat cells).

Contact point analysis. To determine the nucleotide contact points for MNP-1, we carried out methylation interference analysis with the 62-bp fragment (−151 to −90) (Fig. 4). MNP-1 made several contact points with G residues over a 17-bp region from −113 to −97. Methylation of G residues at −113, −111, and −99 on the upper strand (Fig. 4A, lane 2) and at −110, −107, and −97 on the lower strand (Fig. 4B, lane 2) significantly interfered with the binding of MNP-1 to the probe. The binding sites in the region −113 to −107 are outside the contact points for ICK-1A, which makes contacts with G residues at −103, −99, and −95 on the upper strand and −102, −97, and −94 on the lower strand (Fig. 4C) (36). Thus, contact points at −99 (upper strand) and −97 (lower strand) are shared by MNP-1, ICK-1A, and ICK-1B. Neither ICK-1C nor ICK-1D shares those contact points. Interestingly, methylation of the G residues at positions −103 (upper strand) and −102 (lower strand) interfered with the binding of ICK-1A but not MNP-1. These data strongly indicate that MNP-1 is a nuclear factor distinct from the ICK-1 factors which binds to its own distinct *cis*-acting element.

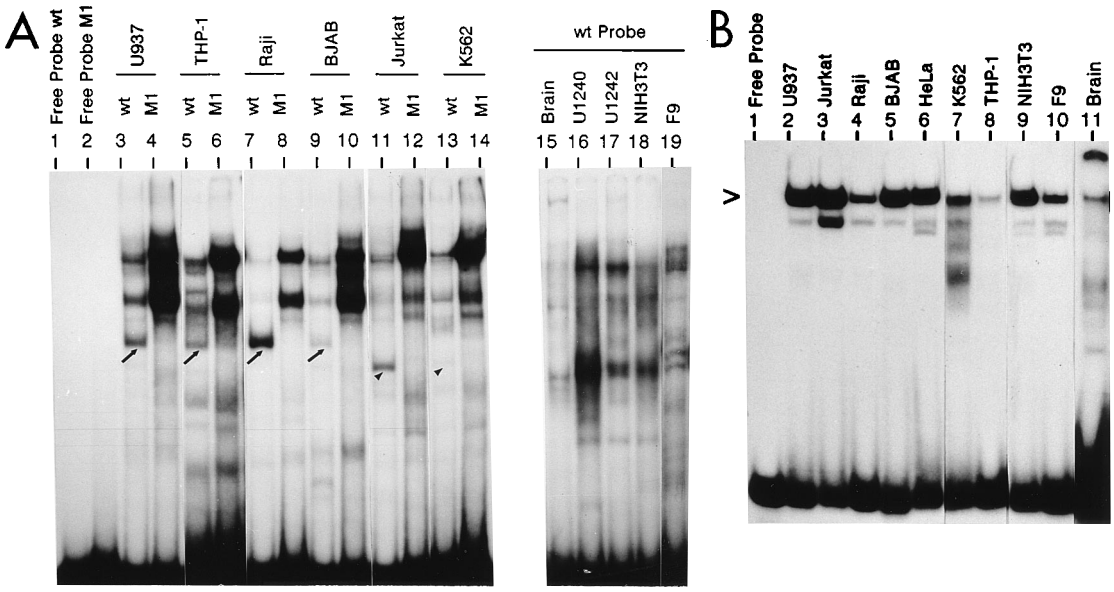


FIG. 5. Cell and tissue type expression of MNP complexes. EMSA of the probe 62wt and the mutant probe 62M1 with crude nuclear extracts from six hematopoietic and five nonhematopoietic cell lines. (A) MNP-1 complexes are indicated by arrows. MNP-2 complexes are indicated by arrowheads. (B) A labeled SP1 consensus double-stranded oligonucleotide complexed with all nuclear extracts to confirm activities of nuclear proteins.

Tissue and cell type expression and mutational analysis of MNP complexes. By using site-directed mutagenesis, a 62-bp double-stranded DNA fragment in which the four upstream contact points for MNP-1 (G residues) were changed to T residues was constructed (62M1; Fig. 4C). In order to determine the tissue and cell type expression of MNP complexes, the 62-bp double-stranded wild-type oligonucleotide (62 wt) and 62M1 were end labeled and allowed to form complexes with nuclear extracts from U937, THP-1, Raji, BJAB, Jurkat, and K562 cells. Shifted complexes were analyzed by EMSA, and the results are shown in Fig. 5A. U937 (lane 3), THP-1 (lane 5), Raji (lane 7), and BJAB (lane 9) extracts showed the presence of the distinctive MNP-1 complex with the probe 62wt. The binding of MNP-1 in these nuclear extracts was abrogated by the presence of the M1 mutation (lanes 4, 6, 8, and 10, respectively). Jurkat cell (lane 11) nuclear extracts showed the presence of the MNP-2 complex with the probe 62wt, and binding was also abrogated by the presence of the M1 mutation (lane 12). K562 nuclear extracts showed only a very faint MNP-2 complex (lanes 13 and 14). In lanes 15, 16, 17, 18, and 19, it can be seen that neither MNP-1 nor MNP-2 could be detected in brain (lane 15), U1240 (lane 16), U1242 (lane 17), NIH 3T3 (lane 18), and F9 (lane 19) cell nuclear extracts. In addition, in Fig. 3, lane 2, neither MNP-1 nor MNP-2 is detected in HeLa S3 nuclear extracts. When the 62M1 mutant probe was used with these nuclear extracts (brain, U1240, U1242, NIH 3T3, F9, and HeLa S3 cell nuclear extracts), the same pattern of bands was obtained as that observed with the probe 62wt, thus confirming the lack of MNP-1 and MNP-2 (data not shown). Figure 5B shows an EMSA with the SP1 consensus sequence oligonucleotide as a probe with these nuclear extracts to show that all were active for nuclear DNA-binding proteins.

The M1 mutation was introduced into the full-length *GOS19.1* promoter fused to the luciferase reporter gene. The mutant pGSP-813M1luc and wt pGSP-813luc constructs were then used to transiently transfect U937 and Jurkat cells. These transfected cultures were subsequently treated with PHA and PMA as previously described. The results were normalized for total protein and expressed as percent relative light units compared with the full-length wt promoter pGSP-813luc (Fig. 6A and B). The M1 mutation completely abolished the activity of the promoter in both noninduced and induced U937 cells (Fig. 6A). In Jurkat cells, the promoter activity was markedly reduced by a factor of 4 but was not abolished (Fig. 6B).

DISCUSSION

Previous work has shown that the *GOS19.1* gene is differentially regulated in T cells and monocytes (15, 19, 28, 34, 37, 42, 52, 54). A DNA sequence, termed the ICK-1 element, which is required for suppression of transcription in nonactivated Jurkat cells and also for induction of expression after cellular activation has been identified (36). This DNA sequence forms four distinct protein-DNA complexes when exposed to nuclear extracts of Jurkat cells. The nuclear factors which form these complexes have been termed ICK-1A, -B, -C, and -D (36). Nomiyama et al. (36) have also presented evidence that at least ICK-1A is required for transcriptional suppression in Jurkat cells.

In this communication, we confirm the presence of a transcriptional silencer for noninduced Jurkat cells in the ICK-1 domain (Fig. 1). Furthermore, we provide additional evidence for a basic difference in the regulation of the *GOS19* genes in T cells and monocytes. Unlike in Jurkat cells, there is no transcriptional suppressor activity of the ICK-1 domain in nonactivated U937 cells (monocytes in Fig. 1C), although this domain is still required for full expression after cellular activation. Thus, the underlying mechanisms for the lack of expression in nonactivated T cells and monocytes appear to be different.

Using EMSA, we investigated the protein-DNA complexes formed from nuclear extracts of Jurkat, U937, and HeLa S3 cells. The DNA probes that were used overlapped in a 30-bp sequence (−119 to −90) which was slightly larger than the defined ICK-1 sequence. By using U937 cell nuclear extracts,

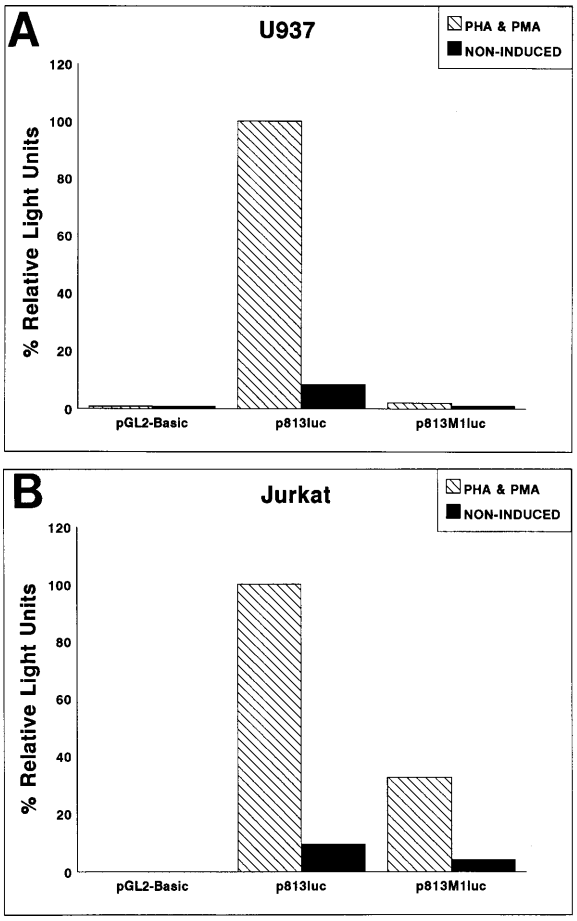


FIG. 6. Transient expression of the full-length *GOS19.1* promoter containing the M1 mutation. The M1 mutation was introduced into the full-length *GOS19.1* promoter and linked to the luciferase reporter in the pGL-2Basic vector (pGSP-813M1luc). The wt construct (pGSP-813luc) and the mutant construct (pGSP-813M1luc) were individually transfected into U937 cells (A) or into Jurkat cells (B) to compare promoter activities with and without the presence of the mutation. Results of luciferase assays are expressed as percent activity relative to the full-length wt promoter pGSP-813luc after normalization for total protein. Black bars, results from nonstimulated cells; striped bars, results after stimulation with PHA and PMA for 8 h.

complexes corresponding to ICK-1A, -B, and -C were identified (Fig. 2). We did not routinely observe ICK-1D complexes under our binding conditions. It has previously been reported that this complex is very sensitive to the amount and composition of nonspecific competitor DNA used in binding assays (36). In addition, a complex corresponding to an upstream CLE0/CK2 site was identified. Our deletion experiments show that this binding site has no effect on promoter activity in either Jurkat or U937 cells (Fig. 1B and C), at least in transient-transfection assays. By using U937, THP-1, Raji, BJAB, Jurkat, and K562 nuclear extracts, two additional faster-migrating complexes, which we have termed MNP-1 and MNP-2, were observed. Competition experiments show that these complexes are much more specific than ICK-1A, -B, or -C. It should be noted that no differences were observed in the pattern of complex formation whether nuclear extracts were prepared from nonactivated or activated cells. We interpret this to mean that whatever the roles of the nuclear factors in suppression of transcription and full expression, the activation must involve modulation of existing proteins and/or the interaction with additional nuclear factors.

The MNP factor(s) makes contact with G residues in the 30-bp domain over a 17-bp region (Fig. 4C). Interestingly, four contact points (two on each strand) between positions −113 and −107 are completely outside the G residue contact points reported for ICK-1A, -B, -C, and -D (Fig. 4C) (36). Furthermore, the two G residue contact points which overlap with the ICK-1 element, at −99 (upper strand) and −97 (lower strand), are shared by ICK-1A and -B. These results strongly suggest a second *cis*-acting element located upstream of and overlapping the ICK-1 element, which we propose to term the MNP site. ICK-1 sites have also been identified in the promoters of the interleukin-3 and granulocyte-macrophage colony-stimulating factor genes. However, these sequences do not contain the upstream sequence which defines the MNP site. With unlabeled synthetic oligonucleotides corresponding to these other ICK-1 sites, competition with the ICK-1A, -B, and -C complexes (at a rather high molar excess of competitor) but not the MNP complexes was possible (data not shown). This further suggests that the MNP complex(es) is a distinct nuclear binding factor(s). The complex nature of the protein-DNA interactions surrounding the ICK-1 and MNP sites suggests the possibility of competition for binding for different factors and possibly cooperative binding. The MNP factor(s) appears to have a higher affinity than any of the ICK-1 factors, and in the presence of the M1 mutation which abrogates the formation of the MNP complexes, the binding abilities of the ICK-1 factors are changed variably. One attractive possible explanation is that in the presence of an MNP factor(s), either or both of the ICK-1A or -B factors may not be able to bind to the ICK-1 site in vivo. It remains to be determined whether this model has any validity for the difference in transcriptional regulation at this domain between Jurkat and U937 cells.

Using nuclear extracts from a wide variety of cell types, we found that the MNP-1 complex was formed with U937, THP-1, Raji, and BJAB nuclear extracts, while a strong MNP-2 complex was formed with Jurkat cell nuclear extracts. The M1 mutation at the MNP site completely abolished the ability of the DNA to form either the MNP-1 or the MNP-2 complex. At this time, we do not know whether the MNP complexes are formed from the same protein(s) or from a family of related proteins. The M1 mutation completely abolished the activity of the *GOS19.1* promoter in U937 cells, suggesting that interaction of the MNP factor(s) with its *cis*-acting binding site is necessary for promoter function in this cell type. However, although the activity was reduced by fourfold in Jurkat cells, it was not completely abolished. This further emphasizes the difference in regulation of *GOS19.1* transcription in the two cell types. Previous work by others (36) has shown that the ICK-1 site alone had little effect on the activity of the thymidine kinase promoter. It would be of interest to investigate the MNP site alone in this way. However, to date we have been unable to inactivate the ICK-1 site without also inactivating the MNP site.

Analysis of transcription factor recognition sequence data bases failed to reveal an identifiable match for the MNP target sequence. We propose that we have identified a novel transcription factor, or family of factors, which is expressed in some hematopoietic cells, but not fibroblasts, epithelial cells, brain tissue, glioblastoma cells, or embryonal carcinoma cells. We believe we have shown here that the MNP factor(s) is crucial for the transcription regulation of the *GOS19.1* gene in T cells and macrophages.

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