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Identification and Characterization of a Novel Enhancer for the Human MCT-1 Oncogene Promoter

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Abstract Cloning and characterization of the promoter region for the MCT-1 oncogene is described. We used luciferase assays to identify *cis*-acting elements responsible for human MCT-1 promoter function. The MCT-1 promoter is TATA-less with a consensus initiator element located at the transcription start site and facilitated by two Sp1 sites that directs basal transcription. Deletion of a region of the MCT-1 promoter (−133 to −122) resulted in significant decrease in luciferase activity, suggesting that this region contains a positive *cis*-acting element. Using mobility shift assays with a 26-mer oligonucleotide, which contains this fragment and its flanking regions, we demonstrated the presence of sequence-specific DNA-binding protein in both Jurkat and Hela nuclear extracts that we designated as LMBF (for lymphoid MCT-1 binding factor). This 26-mer oligonucleotide containing the LMBF binding site is required for maximum transcriptional activity of the MCT-1 promoter. Although the 26-mer oligonucleotide contains a sequence with strong homology to a heat-shock factor consensus, competitive electrophoretic mobility shift assay (EMSA) analysis demonstrated that the binding protein is not a known member of heat shock family. Furthermore, this sequence when placed in reverse orientation downstream of the *luciferase* gene was able to enhance luciferase activity driven by a minimal promoter. These data are consistent with this sequence behaving as an enhancer. Finally, Southwestern blot analysis revealed a 96-kDa protein capable of binding a probe containing the LMBF binding site. J. Cell. Biochem. 90: 68–79, 2003.

Key words: MCT-1; oncogene; promoter; enhancer; transcription factor; DNA-binding protein; heat shock factors; southwestern; EMSA; Jurkat

MCT-1 is a novel oncogene that was discovered as an amplified DNA sequence in the Hut 78 T-cell-line using arbitrarily primed-polymerase chain reaction (AP-PCR) [Prosniak et al., 1998]. MCT-1 maps to chromosome X q22-24, a region frequently amplified in a variety of primary lymphoid neoplasms [Werner et al., 1997; Monni et al., 1998]. Exogenous overexpression of MCT-1 in NIH 3T3 cells shortens the G1 phase of the cell cycle, decreases cell doubling time, and induces the anchorage-independent growth of 3T3 cells on soft-agar [Prosniak et al., 1998]. In stably transfected NIH 3T3 cells, overexpression of MCT-1 correlates with increased kinase activity of cdk4 and cdk6, and increased expression of cyclin D1 [Dierov et al., 1999]. Thus, constitutive expression of MCT-1 results in a strong proliferative signal and is associated with deregulation of protein kinase-mediated G1/S phase checkpoint [Dierov et al., 1999]. We have previously shown that MCT-1 expression correlates with IL2-dependent status of T-cell leukemia/lymphoma cell lines; high protein levels are observed in IL-2-independent lines, and low levels are seen in IL2-dependent cells [Dierov et al., 1999]. We also observed elevated expression of MCT-1 in the aggressive B-cell lymphoma, diffuse large B-cell lymphoma [Shi et al., 2003]. In contrast, chronic lymphocytic leukemia (CLL),

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an indolent lymphoproliferative disorder exhibited barely detectable amounts of MCT-1 protein [Shi et al., 2003].

MCT-1 is predominantly localized to the cytoplasm in lymphoid cells and protein levels of MCT-1 are apparently stable throughout the cell cycle [Herbert et al., 2001]. This coincides with a long half-life (~19 h) of MCT-1 protein in Jurkat and MT-2 T-cell leukemia cells [Herbert et al., 2001]. A potentially important feature of MCT-1 is an RNA binding domain (PUA) in the carboxy terminal half of the molecule [Herbert et al., 2001]. The PUA domain is associated with the translational machinery and has been detected in a family of eukaryotic proteins with homologies to the translation initiation factor eIF1/SUI1 [Aravind and Koonin, 1999; Sheikh et al., 1999]. Thus, MCT-1 may be involved in translational regulation of gene expression.

Interestingly, many of the lymphoid cell lines with overexpression of MCT-1 protein have been found not to have MCT-1 gene amplification [Prosniak et al., 1998]. Furthermore, no point mutations have so far been identified in human tumors [Shi et al., 2003]. These observations suggest that regulation of MCT-1 expression is complex and may involve both transcriptional and post-transcriptional mechanisms. In order to identify and characterize the *cis*-acting elements responsible for regulating the MCT-1 gene expression, we have cloned the 5′-flanking region of MCT-1. Furthermore, we have identified a 26-mer sequence containing a potential enhancer element required for maximal transcriptional activity. Finally, we demonstrated using Southwestern analysis that a putative novel 96-kDa transcription factor can specifically bind to this 26-mer DNA element.

MATERIALS AND METHODS

Cell Culture and Heat Shock Treatment

T-cell lymphoma cell lines Jurkat, Hut 78, C91PL, and C10MJ were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and grown in RPMI-1640 supplemented with 10% fetal calf serum (Life Technologies, Inc., Gaithersburg, MD). Heat shock of Jurkat cells was performed by placing cell containing flasks in 42°C water bath for 30 min and cells were incubated in 37°C, 5% CO_2 incubator for 1 h, and then cell nuclear extract was isolated as below.

Plasmid Construction

5′-Flanking region of the *MCT-1* gene (1,338 bp; from −1301 to +37) was PCR amplified from both Jurkat and PBL genomic DNA using primers with engineered KpnI and HindIII sites. Both PBL and Jurkat contained identical 5′-flanking regions of the *MCT-1* gene. The 3′-end of fragment contains a 39 nt sequence of exon1 in MCT-1. The 1.34-kb PCR product was cloned in the sense orientation into the Kpn I and Hind III sites of the pGL3-basic luciferase reporter vector (Promega, Madison, WI). Progressive 5′-deletion of MCT-1 promoter constructs were engineered by unidirectional cloning of PCR fragments from the MCT-1 promoter into the Kpn I and Hind III sites of the pGL3-basic vector. All of the constructs were sequence-verified. These PCR fragments (except F12) were generated using a common reverse primer (Rev 1) and 11 different forward primers (Table I). Fragment12 (F12) was produced by forward 3′-primer and a reverse primer (Rev 2) that deleted 141 nt in the 3′-end of the promoter. The forward and reverse

TABLE I. Primers Used for Generating MCT-1 Promoter Deletion Mutants

Fwd 1	5′-GAGCGGTACCAGGTTTTTAAATTTTT-3′	(−1301 to −1284)
Fwd 2	5′-AGGACTTTGGTACCAGCCTGAG-3′	(−730 to −722)
Fwd 3	5′-GGTTATAAGGTACCAAACAGGATG-3′	(−453 to −442)
Fwd 4	5′-TTCAGGTACCCAAGCGCTGTA-3′	(−423 to −412)
Fwd 5	5′-AATAGGTACCCTGTATTTTCATTTGC-3′	(−315 to −299)
Fwd 6	5′-TAGGTAGGTACCAATCCATTCGGT-3′	(−226 to −214)
Fwd 7	5′-CTAGGTACCTCGTTTACTTTGACGATA-3′	(−174 to −156)
Fwd 8	5′-GATAGGTACCAACGAAGCACGG-3′	(−150 to −138)
Fwd 9	5′-CACGGTACCATCTTGACCCCT-3′	(−133 to −121)
Fwd 10	5′-TCTTGGTACCTTGAGGAACCCG-3′	(−122 to −110)
Fwd 11	5′-GCCAGGTACCATAAATGCC-3′	(−93 to −84)
Rev 1	5′-GGAAGCTTTTAGGCAACCGG-3′	(+37 to +25)
Rev 2	5′-CGTAAGCTTCGGGTTCCTCAA-3′	(−122 to −110)

primers contain a Kpn 1 and a Hind III restriction sites, respectively that were underlined as shown. The numbers indicate sequence distances from the transcription start site. The PCR reaction was programmed for 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and it was repeated 34 cycles.

Transient Transfection and Luciferase Reporter Gene Assay

All the pGL3-promoter constructs were transfected into Jurkat cells by electroporation with a Bio-Rad 'Gene Pulser II' (Bio-Rad, Richmond, CA,) using the method described previously [Gartenhaus et al., 1991] with minor modifications. The Jurkat cells were resuspended in the culture medium to make a cell suspension with a concentration of 8×10^7 cells/ml. Then 0.25 ml of the cell suspension was transferred to a 0.4 cm transfection cuvette (Bio-Rad) and 20 μg of a pGL3-construct and 20 μg of pSV-β-galactosidase control vector were added. The cell suspension and plasmid DNA were mixed gently and incubated on ice for 10 min. A single pulse of 975 μF, 250 V was applied to each sample cuvette, followed by an additional 10 min incubation on ice. The cells were then transferred into 25 cm^2 flasks with 15 ml complete RPMI-1640 medium and cultured for 48 h. Luciferase activity was assayed by using a luciferase assay kit according to the manufacturer's instructions (Promega). Cell extracts were prepared in 500 μl 1× reporter lysis buffer. The lysates were vortexed for 10 s and centrifuged at 13,000*g* for 2 min to pellet the cell debris, and supernatant was transferred to a new tube. Twenty microliters of the cell lysate was transferred to a 96 well plate and luciferase activity was detected in a Microlumat LB96P Luminometer (Perkin Elmer, Wallac, Inc., Gaithersburg, MD). β-galactosidase activity of the cell lysate served as an internal control for transfection efficiency and was analyzed using β-galactosidase enzyme system as described by the manufacturer (Promega). Luciferase activity was normalized to β-galactosidase activity. Each transfection was carried out in duplicate. The luciferase reporter assay experiments were repeated at least three times. The results are reported as mean ± standard error (SE).

Electrophoretic Mobility Shift Assay (EMSA)

Jurkat nuclear extracts were prepared as described previously [Yan and Hung, 1991]. Briefly, 10^7 cells were harvested and washed twice with cold PBS. The pelleted cells were resuspended in 400 μl cold buffer A (10 mM HEPES pH 7.9, 10 m KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF). Cells were allowed to swell on ice for 10 min and then vortexed for 10 s. Cells were washed twice with 400 μl cold buffer A. The pellet was resuspended in 100 μl cold buffer C (20 mM HEPES pH 7.9, 25% glycerol, 420 m NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) and incubated on ice for 20 min for high-salt extraction. Cellular debris was pelleted by centrifugation and supernatant was stored at −70°C. Hela cell nuclear extract, goat polycolonal anti-HSF1, and anti-HSF2 were purchased from Santa Cruz (Santa Cruz, CA). Oligonucleotides Sp1 (upper strand, 5′-ATTCGATCGGGGCGGGGCGAGC-3′) and AP1 (upper strand, 5′-CGCTTGATGAGTCAGCCGGAA-3′) were commercially obtained (Promega). EMSA was performed using the Gel shift assay kit according to the protocol provided by manufacturer (Promega). Briefly, our 26-mer oligonucleotide (upperstrand, 5′-GGAAGAATCTTGACCCTTTGAGGAAC-3′) was annealed by mixing equimolar amounts of the forward and reverse oligonucleotides, heating to 80°C for 5 min, and allowing the oligonucleotides to cool slowly down to room temperature. Heat shock element (HSE) oligonucleotide (5′-CTAGAAGCTTCTAGAAGCTTCTAG-3′) was annealed under similar conditions [Goodson et al., 1995]. The annealed DNA fragments were labeled at the 5′-ends with [γ-^{32}P] ATP and unincorporated nucleotides were removed by G-25 spin column. For a typical binding reaction, we mixed 1× binding buffer, 5 μg of nuclear extract protein, and a 100-fold excess of appropriate cold oligonucleotide or 1 μg of antibody, and incubated the reaction at room temperature for 10 min. End-labeled DNA (0.07 pmol) was added to the mixture and incubated for another 20 min at room temperature. The binding mixture was analyzed using a native 4% polyacrylamide gel (acrylamide/bisacrilamide ratio, 29:1) containing 0.5× TBE and 2% glycerol. The gel was then dried and autoradiographed with intensifier screens at −70°C.

Southwestern Hybridization

Southwestern analysis was performed according to the method of [Yan and Hung, 1991] with minor variations. Briefly, 50 μg of Jurkat,

HeLa, Hut78, C91PL, and C10MJ cell nuclear extract were separated on 12% SDS–PAGE at room temperature. The proteins were transferred to Hybond nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) by using a semidry electroblotter (Bio-Rad, Hercules, CA). The blots were blocked in blocking buffer (5% non-fat dry milk in 10 mM HEPES, pH 7.8) for 1 h at room temperature, and then hybridized with 1,000,000 cpm/ml of ^{32}P-labled 26-mer-oligo probe in hybridization buffer (10 mmol HEPES, pH 7.9, 50 m NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 0.25% non-fat dry milk) overnight at room temperature. The nitrocellulose membranes were washed with washing buffer (10 mM HEPES, pH 7.9, 200 m NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.25% non-fat dry milk) with three changes for 1 h each at room temperature. The blots were wrapped in plastic and exposed to phosphor imaging screen cassette at -70°C and scanned with the Storm 860 phosphoimager machine (Molecular Dynamics, Sunnyvale, CA). The membrane was stripped with stripping buffer (100 mMol β-mercaptoethanol, 2% SDS, 62.5 mMol Tris–HCl pH 6.7) at 50°C for 30 min, then washed for 10 min $\times 2$ in TBST buffer and then reprobed with 1,000,000 cpm/ml of ^{32}P-labled Sp1-oligonucleotide under the same conditions as described above.

Insertion of a 26 mer-oligo Downstream of the *Luciferase* Gene in pGL3-F10 Vector

The annealed 26-mer was inserted in reverse orientation at the Sal I and BamH I sites located downstream of the *luciferase* gene in the pGL3-F10 plasmid (Fig. 5A). The pGL3-F10-26-mer construct, pGL3, and pGL3-F10 vectors were transfected into Jurkat cells and the luciferase activities of these constructs were determined as described above. Each transfection was performed in duplicate. The luciferase reporter gene assay experiments were repeated three times. The results are reported as mean ± SE.

RESULTS

Cloning the 5′-Flanking Region of the Human MCT-1 and Analyzing the Putative Transcription Factor Binding Sites

The 1.34 kb of 5′-flanking region of *MCT-1* gene was cloned and sequenced. The putative transcription factor binding sites in the 5′-franking region were detected by MatInspector and TFsearch program (see Fig. 1). The transcription start site of MCT-1 was determined by 5′-RACE as previously reported [Prosniak et al., 1998]. A TATA box was not found within the −40 bp relative to the transcription initiation site. However, a consensus initiator element (Inr, TTCAGCT, shadowed in Fig. 1) was identified at the transcription start site, which was composed of a central CA, a T in +3 position and surrounded by several pyrimidines as previously reported [Smale, 1997]. Two GC boxes (Sp1 box) at −10 and −65 bp and two CCAAT boxes for transcription factors NF–Y (−223 bp) and CEBPB (−210 bp) were identified. GC boxes serve to modulate basal transcription of the core promoter and operate as enhancer sequences. We have also identified consensus sequences for IK2, CREB, TTF1, AP4, and HSTF2 in the proximal region of the promoter. There was no obvious downstream promoter element (DPE), containing the consensus sequence A/GGA/TCGTG (usually positioned at +30) [Burke and Kadonaga, 1997].

Functional Deletion Mapping of the Human MCT-1 Promoter

The deletion constructs of the MCT-1 promoter placed upstream of the *luciferase* reporter gene were used to define the minimal promoter sequences for the maximal and basal transcription activities (Fig. 2). A series of deletion mutants covering the 5′-flanking region from −1,304 bp (pGL3-F1) to −133 bp (pGL3-F9) with common 3′-end (+37), did not significantly decrease luciferase activity in Jurkat cells. However, further deletion of 11 nucleotides ATCTTGACCCT from −133 (pGL3-F9) to −122 (pGL3-F10), reduced luciferase activity approximately 13-fold, indicating that these 11 nucleotides are critical for MCT-1 promoter maximal transcription activity. The next deletion construct (promoter fragment F11 with sequences from −93 to +37) provided only basal/constitutive transcription activity. Finally, deletion of the sequences from −119 to +37 (construct F12) completely abolished the promoter activity, indicating that the sequence between −119 and +37 contained the essential core promoter elements.

The 26-mer Oligonucleotide Binds a Putative Transcription Factor

To determine whether the 11 bp fragment ATCTTGACCCT binds a protein that may be

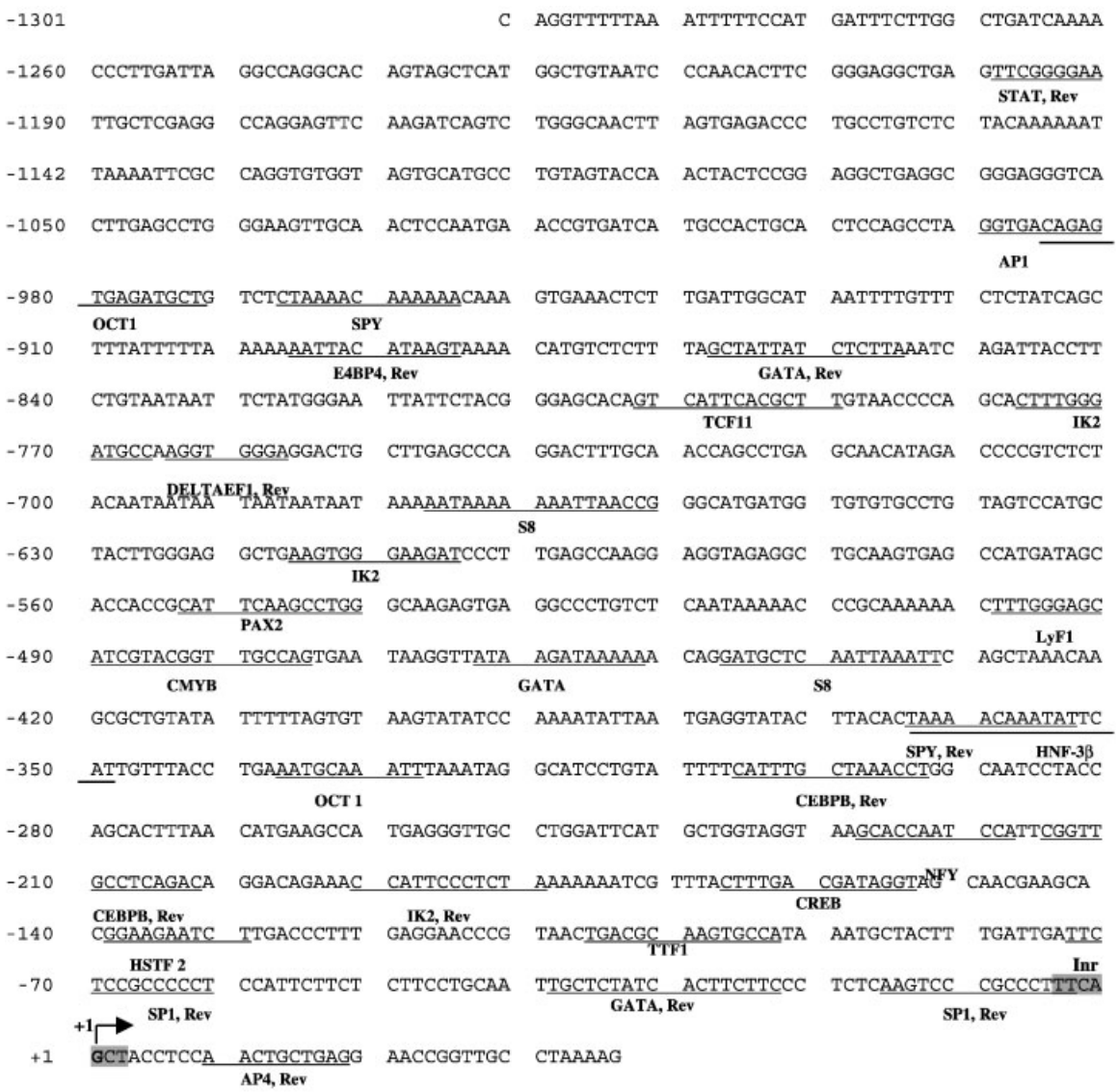

Fig. 1. Nucleotide sequence of the 5′-flanking region of the human *MCT-1* gene. A total of 1,338 bp of MCT-1 5′-flanking region was cloned and sequenced. The mRNA initiation site is indicated by a horizontal arrow and designated with +1 in bold letter. Sequence is numbered relative to the transcription start site. Putative transcription binding sites are underlined and the binding transcription factors are shown below the lines. The orientations of the binding sites are indicated as antisense sequence with 'Rev' and as sense if not marked.

involved in regulation of MCT-1 promoter activity, we performed EMSAs. As a probe we used a radiolabeled 26-mer oligonucleotide (upper strand, 5′-GGAAGAATCTTGACCCTTTGAGGAAC-3′), which contained the crucial 11-mer sequence (bolded) and 5′- and 3′-flanking sequences. The probe was incubated with Jurkat and Hela cell nuclear extracts, and complexes were separated as described in Materials and Methods. The intensity of the bands on the top part of the gel (Fig. 3A) increased proportionally with the addition of nuclear extract and decreased when competing cold 26-mer was added thus indicating specificity of DNA-protein interaction. These bands intensities were not affected by two irrelevant oligonucleotides (AP1 and Sp1) when they were added at the same molar excess as the 26-mer fragment (Fig. 3B). These results further corroborated the binding specificity of the 26 mer–protein complexes.

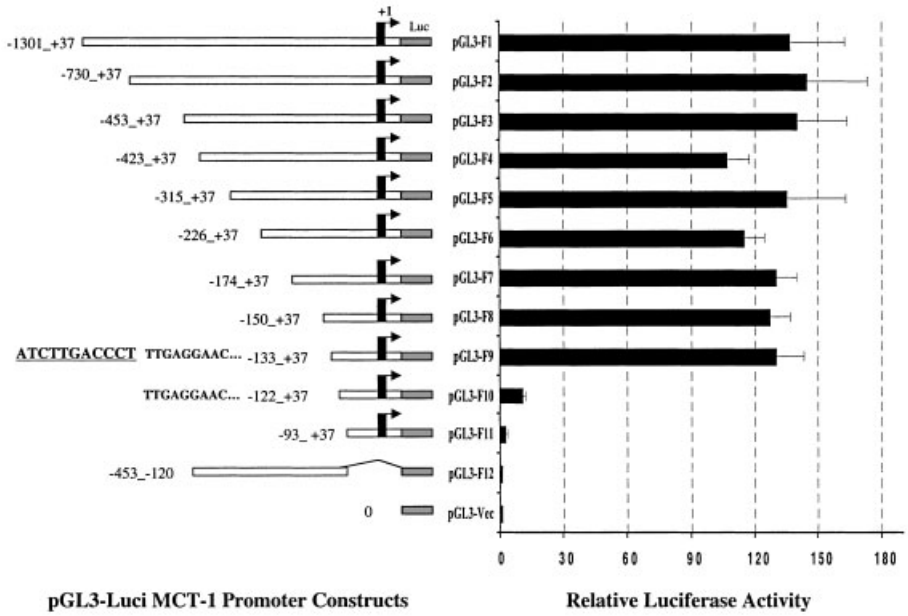

Fig. 2. Functional deletion mapping of the MCT-1 promoter. Depicted on the left side of the figure are the MCT-1 promoter deletion mutants cloned upstream of the Lucifer's gene in the basic pGL3 vector. The numbers on the left of the each promoter deletion construct refer to the beginning and end position of the promoter fragments. The transcription start site in the promoter fragments is indicated by an arrow and +1. Each construct was transiently co-transfected with the pSV-β-galactosidase vector into Jurkat cells and luciferase activities are depicted in graphic form on the right. Transfections were carried out in duplicate and individual experiments were repeated three times. Luciferase activity value was normalized to β-galactosidase activity and presented as fold increase relative to the basic PGL3 vector. By deletion of 11 nucleotides ATCTTGACCCT in the 5′-end of pGL3-F9, which is shown on the left side with the underlined sequence, luciferase activity was dramatically reduced.

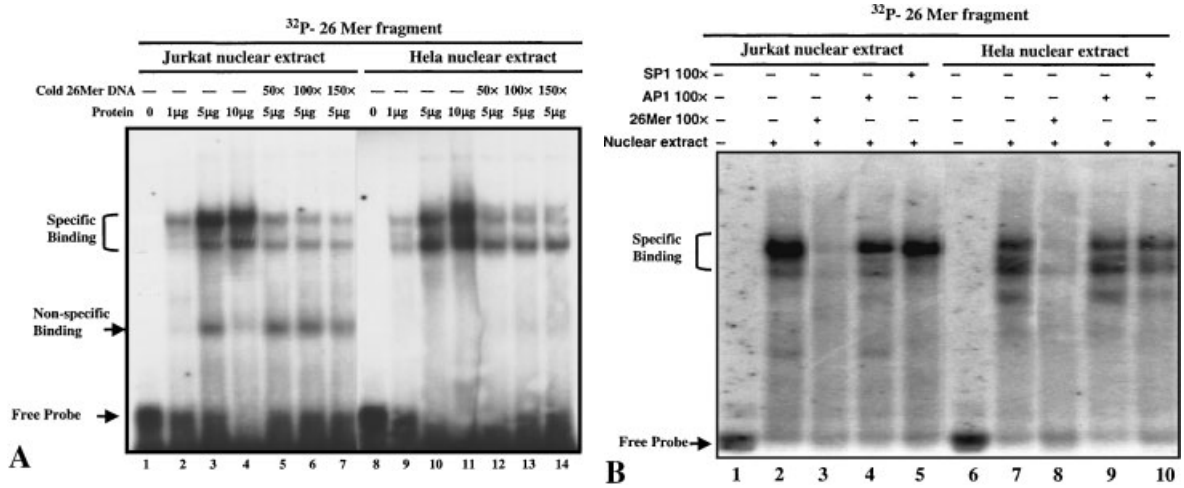

Fig. 3. Electrophoretic mobility shift assay (EMSA) analysis of protein binding to 26-mer fragment. **A**: The EMSA was performed by incubating 0.07 pmol of 5′-ends labeled 26-mer fragment with either Jurkat or Hela nuclear extracts. Different amounts of nuclear protein 0, 1, 5, 10 μg were added to the binding mixture, which correlated with increased specific band intensity. The competition was performed in the presence of either 50, 100, 200-fold molar excess of the cold 26-mer fragment. The cold 26-mer oligonucleotide was able to successfully compete away the specific binding but not the non-specific bands. **B**: Competition assay was carried out using 100-fold excess of the cold 26-mer oligonucleotide and two irrelevant oligonucleotides, AP1 and Sp1. Specific protein–DNA complexes could be competed away by the 26-mer oligonucleotide, but not the AP1 and Sp1 oligonucleotides.

Identification of a Putative Transcription Factor Interacting With the 26-mer Oligonucleotide Containing the LMBF-Binding Site

Southwestern analysis was performed by Western-blotting 50 μg of Jurkat and Hela cell nuclear extract to Hybond nitrocellulose membrane, which was then probed with 10^6 cpm/ml of ^{32}P-labeled 26-mer probe. The results revealed a single strong band in both Jurkat and Hela cells with a molecular weight of approximately 96 kDa (Fig. 4A). Control experiments were done by stripping the membrane and reprobing with ^{32}P-labeled Sp1 oligonucleotide. In this experiment, we detected a single band with the expected molecular weight ~106 kDa, which was consistent with binding of the p106 kDa subtype of Sp1 (data were not shown). Additional experiments of loadind 50 μg of Jurkat, Hut78, C91PL, and C10MJ cell nuclear extracts on Southwestern membrane demonstrated a correlation between the degree of 26-mer oligonucleotide binding with the 96 kDa polypeptide and the level of MCT-1 RNA present in different lymphoid cell lines (Fig. 4B).

The Putative Transcription Factor Binding Sequence Possesses Enhancer Like Activity

Formation of DNA–protein complexes with the 26-mer oligonucleotide may be indicative of enhancer-like activity of this sequence. To test potential enhancer activity of the 26-mer oligonucleotide, we inserted it in the reverse orientation downstream of the *luciferase* gene in the pGL3-F10 construct (Fig. 5A). The pGL3-F10-26-mer oligonucleotide, pGL3, and pGL3-F10 constructs were transfected into Jurkat cells, and corresponding luciferase activities were determined. The results (Fig. 5B) showed that luciferase activity of pGL3-F10-26-mer construct was increased over threefold compared to pGL3-F10, containing only core promoter elements. Thus, the 26-mer fragment acts as an enhancer when cloned downstream of a reporter gene.

The Putative Transcription Factor is not a Heat Shock Transcription Factor (HSTF)

High-stringency (homology >90%) database search for potential transcription factors that might bind to the 26-mer sequence yielded only a HSTF consensus sequence, the HSE (Fig. 6A). We asked whether the protein bound to our 26-mer fragment is a HSTF. To address this ques-

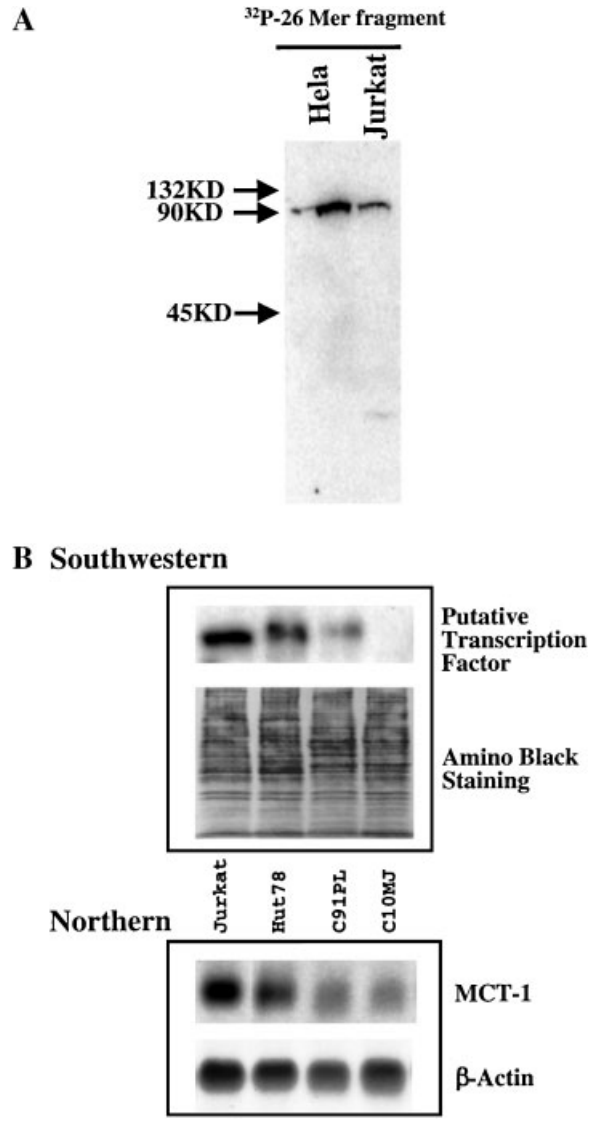

Fig. 4. Identification of a potential trans-acting factor that binds to the 26-mer fragment. **A**: Southwestern analysis was performed as described in Materials and Methods. Fifty micrograms of Jurkat and Hela cell nuclear extracts were subjected to SDS–PAGE. After incubating in blocking buffer (5% non-fat dry milk in 10 mM HEPES, pH 7.8), the blot was hybridized with 1,000,000 cpm/ml of ^{32}P-labeled 26-mer probe. A 96-kDa polypeptide band is shown to interact with the 26-mer probe. As a control we utilized an Sp 1 oligonucleotide. By stripping the membrane and then hybridizing with ^{32}P-labeled Sp1 oligonucleotide, we obtained a single 106-kDa band consistent with Sp1 binding. **B**: Southwestern analysis was carried out using nuclear extracts from Jurkat, Hut78, C91PL, and C10MJ different lymphoid cell lines. There was increased DNA–protein complex formation as demonstrated by the increased intensity of the 96-kDa polypeptide band that correlated with increased levels of MCT-1 RNA. Equal protein loading was verified by amino black staining.

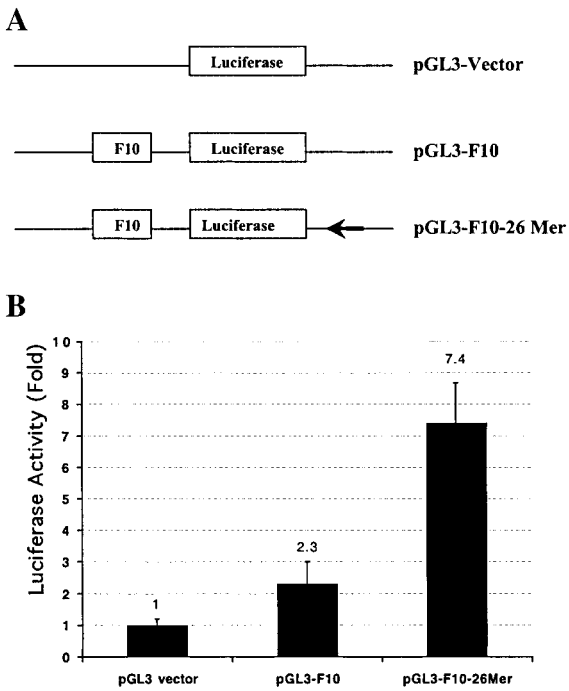

Fig. 5. Enhancer activity of 26-mer fragment. **A**: Schematic view of 26-mer inserted into pGL3-F10. In order to test possible enhancer function of 26-mer fragment, the annealed 26-mer sequence was inserted downstream of the *luciferase* gene in the pGL3-F10 plasmid. The arrow demonstrates the insertion orientation. **B**: The pGL3-F10-26-mer construct, pGL3, and pGL3-F10 vectors were transiently transfected into Jurkat cells, respectively and the luciferase activities of these constructs were determined as described in Materials and Methods. Each transfection was performed in duplicate. Individual experiments of luciferase reporter assays were repeated three times. The results are reported as mean ± SE. Luciferase activities of the constructs were shown as solid bars. The fold enhancements are expressed relative to the basal activity of pGL3 assigned a value of one. There was a greater than threefold increase in the activity of pGL3-F10-26-mer compared to pGL3-F10 and over sevenfold compared to pGL3.

tion, we prepared nuclear extracts from both untreated and heat shocked Jurkat cells, and performed EMSAs with both extracts and either the 26-mer fragment or HSE oligonucleotide probes. HSE oligonucleotide was synthesized as previously reported [Goodson et al., 1995] using the sequence CTAGAAGCTTCTAGAAGCTT which contains four perfect inverted 5′-NGAAN-3′ repeats and is capable of forming self-complementary strands (Fig. 6A).

With nuclear extract prepared from uninduced Jurkat cells, the HSE probe produced a single retarded band. This band was competed away by 100-fold molar excess of cold HSE or 26-mer oligonucleotides, but not by an irrelevant Sp1 oligonucleotide. Interestingly, its mobility was not further retarded by the anti-HSF1, HSF2 antibodies (Fig. 6B), indicating that this HSE-protein complex did not include HSF1 or HSF2. In contrast, HSE incubated with nuclear extract from heat shocked Jurkat cells produced several further shifted bands with much stronger signals (Fig. 6B). Formation of these bands could be blocked only by cold HSE but not the 26-mer fragment. Additionally, their mobility was further retarded by anti-HSF1 antibody (supershift), but not by the anti-HSF2 antibody (Fig. 6B). Since HSFs are activated and acquire DNA binding ability after heat shock treatment, we concluded that HSF1 is the main activated transcription factor that binds to HSE under our heat shock conditions (Fig. 6B).

As we demonstrated earlier (Fig. 3A,B), three specific DNA–protein complexes were observed in the EMSA with nuclear extracts from untreated Jurkat and Hela when probed with the 26-mer fragment. These bands could be blocked by 100-fold molar excess of cold 26-mer fragment but not by two irrelevant oligonucleotides (AP1 and SP1). We confirmed these results (Fig. 6C) and showed that the 26-mer–protein complexes were competed away by 100-fold molar excess of HSE oligonuceotide, while both anti-HSF1and anti-HSF2 antibodies had no effect on the complex mobility. These data indicated that the DNA binding protein from untreated Jurkat nuclear extract, which binds to the 26-mer fragment, was neither HSF1 nor HSF2, even though the binding consensus for these transcription factors is present in our 26-mer oligonucleotide. Moreover, the binding pattern of 26-mer fragment did not depend on the heat shock treatment, nor was its mobility changed after addition of anti-HSF1 or HSTF2 antibodies (Fig. 6D). Apparently, neither HSF1 nor HSF2 were the binding partners to the 26-mer DNA sequence.

DISCUSSION

RNA polymerase II is responsible for transcription of most protein-coding genes in eukaryotic cells. Core promoters serve as assembly sites for the transcription complex and are located in the immediate vicinity of the initiation site. Core promoters, responsible for basal transcription activity, may contain either a TATA box consensus or an initiator element ± DPE. Non-core promoter sequences are

involved in regulating the promoter transcription activity, and in many cases include SP1 sites and CCAAT boxes in the proximal promoter region (−50 to −200 bp). Enhancer and silencer elements, which may reside further away either upstream or downstream, play important roles in regulation of gene transcription [Strachan and Read, 1999].

As we have established here, the MCT-1 promoter is a TATA-less promoter, which con-

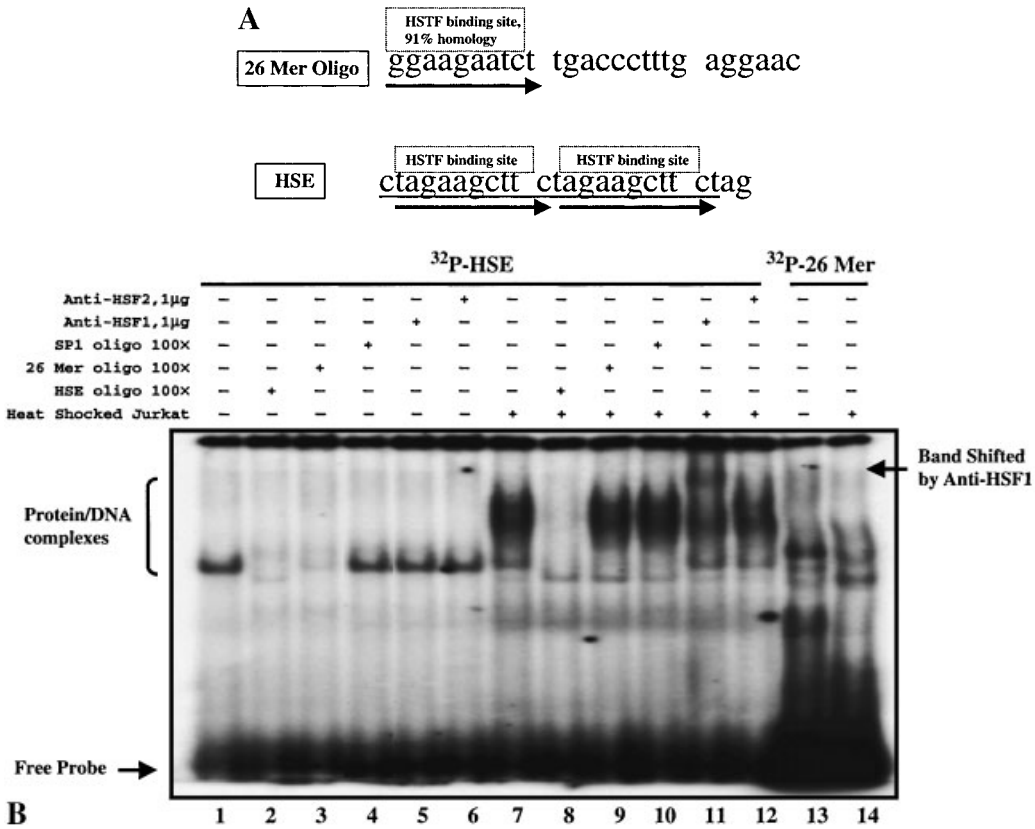

Fig. 6. The DNA-binding protein is not a heat shock transcription factor (HSTF). **A**: Sequence comparison of 26-mer fragment with the consensus heat shock element (HSE). The 26-mer fragment contains a HSF binding site with 92% homology that has two GAA repetitions and one inverted GAA (ctt). HSE oligonucleotide was synthesized with four perfect inverted 5′-NGAAN-3′ repeats and was capable of forming complementary strands by itself. **B**: Different binding pattern of HSE by nuclear extracts from non-heat shocked and heat shocked Jurkat cells. Jurkat cells were heat shocked by placing cell containing flasks in 42°C water bath for 30 min and then incubated in 37°C, 5% CO_2 incubator for 1 h before nuclear extract was isolated. The EMSA conditions were described in Materials and Methods. Five micrograms of nuclear extract protein from heat shocked or non-heat shocked Jurkat cells was used in EMSA assay. A single retarded band on the EMSA was produced by incubating with non-heat shocked nuclear extract (**lanes 1 to 6**). This band was competed away by 100-folds excess molar of unlabeled-HSE (lane 2) or 26-mer oligonucleotide (lane 3), but could not be competed by an irrelevant Sp1 oligonucleotide (lane 4), nor was it supershifted by the anti-HSF1, HSF2 antibodies (lane 5, 6). When HSE incubated with heat shocked nuclear extract, it produced several retarded bands with more intense signals. These bands could only be competed away by cold HSE (**lane 8**) but not the 26-mer fragment (**lane 9**), Sp1 (**lane 10**) and also were supershifted by anti-HSF1 antibody (**lane 11**) but not the anti-HSF2 antibody (**lane 12**). **C**: EMSA analysis of 26-mer probe incubated with non-heat shocked Jurkat nuclear extract. Three specific protein-26-mer oligonucleotide complexes were observed and these bands could be competed away by either 100-fold cold 26-mer fragment or cold HSE (**lanes 2, 3**), but not by two irrelevant oligonucleotides, AP1 and Sp1 (**lanes 4, 5**). The complexes were not supershifted by either anti-HSF1 or HSF2 antibodies (**lanes 6, 7**). **D**: EMSA analysis of 26-mer oligonucleotide incubated with heat shocked Jurkat nuclear extract. **Lane-1**, non-heat shocked Jurkat nuclear extract without competitor; **lane-2**, non-heat shocked Jurkat nuclear extract, plus 1 µg of anti-HSF1; **lanes 3–5**, heat shocked Jurkat nuclear extract, plus 100-fold molar excess of cold 26-mer, 1 µg of anti-HSF1 and 1 µg of anti-HSF1, respectively; **lanes 6–7**, labeled HSE incubated with heat shocked Jurkat nuclear extract plus 1 µg of anti-HSF1 (lane 7). The EMSA pattern of the binding complexes to the 26-mer fragment incubated with heat shocked Jurkat nuclear extract did not change comparing to that of incubating with non-heat shocked nuclear extract. Adding either anti-HSF1or anti-HSF2 antibody to the binding mixture did not supershift the bands. Only the HSE oligonucleotide was able to be supershifted when incubated with heat-shocked lysate and ant-HSF1 antibody.

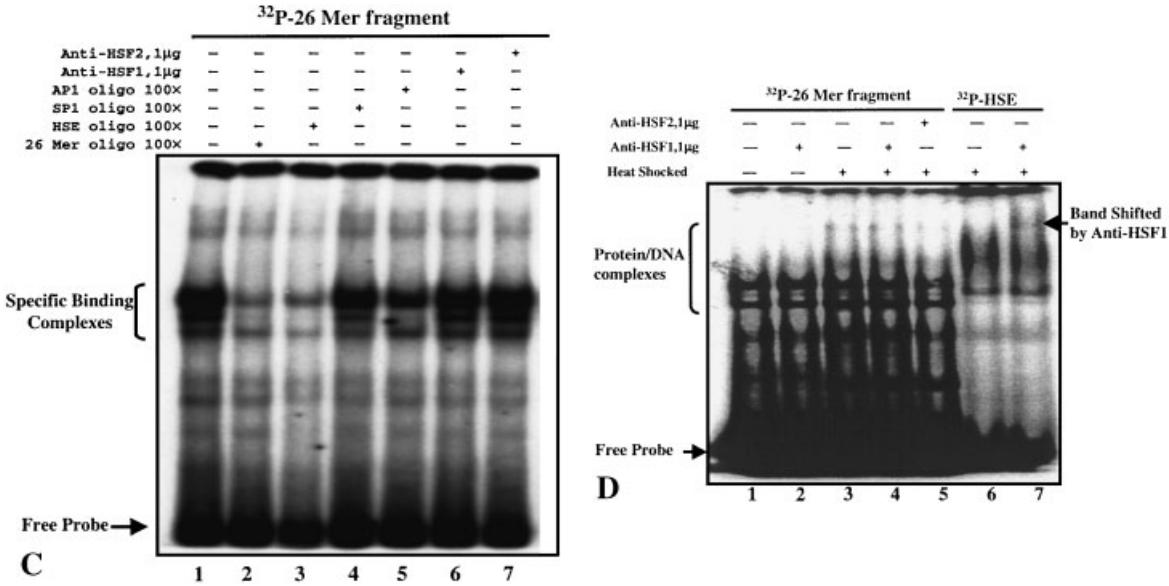

Fig. 6. (*Continued*)

tains an initiator (Inr) consensus around the transcription start site. In TATA-less promoters, the Inr is functionally analogous to TATA, directing basal transcription by RNA polymerase II and determining the precise site of transcription initiation [Means and Farnham, 1990; Kaufmann and Smale, 1994]. The Inr is recognized by TFIID, but physical interactions are mediated by some of the TBP (TATA binding protein) associated factors, or TAFs [Means and Farnham, 1990; Kaufmann and Smale, 1994]. When multiple Sp1 binding sites are located close to the Inr, transcription initiation is more efficient [Abrescia et al., 2002]. As we have demonstrated (Fig. 1), the MCT-1 promoter contains two Sp1 sites at −10 and −65 bp and two CCAAT boxes at −210 bp (CEBPB) and −223 bp (NF−Y), respectively. GC boxes serve to modulate basal transcription of the core promoter and operate as enhancer sequences [Smale, 1997]. For TATA-less promoters, multiple Sp1 sites close upstream of the transcription start site greatly enhance the Inr's strength [Smale, 1997]. DPEs are important for Inr-dependent promoters, where they function in part by increasing TFIID−promoter complex formation and/or stability through direct interactions with TAF's. The DPE exists in many *Drosophila* promoters as commonly as a TATA box. However, in higher eukaryotes the DPE has been identified by functional analysis thus far in only two human TATA-less promoters, that of the IRF-1 and CD30 receptor genes [Abrescia et al., 2002]. No obvious DPE consensus was found in the vicinity of downstream region in MCT-1 promoter (Fig. 1).

By functional deletion mapping, we found that a 170-bp promoter fragment from −133 to +37 is the minimal sequence required for transcription activity in Jurkat cells. We also identified an 11 bp sequence, ATCTTGACCCT which is critical for maximal transcription activity of the MCT-1 promoter (Fig. 2). To explore the possibility that a transcription factor binds to this sequence, we examined complex formation by EMSA using nuclear extracts from both Jurkat and Hela cells, and the 26-mer oligonucleotide containing the critical 11 bp fragment with flanking sequences. We show here that the 26-mer DNA can form complexes with nuclear extract preparations from both Jurkat and Hela cells. These protein−DNA complexes are specific as shown by competition assays when only the 26-mer but not Sp1 or AP1 consensus probes can act as a competitor for complex formation (Fig. 3A,B). We have determined the molecular weight of this putative transcription factor (∼96 kDa) by Southwestern analysis (Fig. 4A) and have identified this protein in both Jurkat and Hela cells (Fig. 4A). There was also an observed increase in the specific binding between the 26-mer oligonucleotide and the 96 kDa polypeptide in those lymphoid cell lines with increased levels of MCT-1 RNA (Fig. 4B). These findings suggest that the observed in vitro interaction between the 26-mer oligonucleotide

containing the LMBF sequence and the 96 kDa polypeptide has functional relevance.

Since the 26-mer sequence is essential for maximal MCT-1 promoter activity and also binds a 96 kDa protein, it appears to act as an enhancer. Enhancers are positive regulatory elements that increase the basal level of transcription regardless of their orientation or their distance from the genes that they regulate [Blackwood and Kadonaga, 1998]. In our experiments, the 26-mer fragment significantly increased luciferase activity when cloned in reverse orientation downstream of a reporter gene (Fig. 5), demonstrating enhancer activity.

The search for possible binding partners (Matinspector, 90% homology threshold) revealed that only the HSFconsensus (HSE) shared homology with the 26-mer sequence (Fig. 6A). HSTFs are sequence-specific DNA binding proteins that bind tightly to multiple copies of nGAAn motifs (HSEs) in the promoter regions of heat shock genes [Rabindran et al., 1991; Nakai and Morimoto, 1993]. In vertebrates, four members of the HSF family (HSF1, HSF2, HSF3, and HSF4) have been identified [Rabindran et al., 1991; Nakai and Morimoto, 1993; Nakai et al., 1997; Schuetz et al., 1999]. HSF1 and HSF2 are ubiquitously expressed as inert monomers and dimers respectively, which localize in both cytoplasm and nucleus in unstressed cells. Upon activation, these two HSFs trimerize and accumulate in the nucleus, where they bind to HSE and activate heat shock gene expression. HSF1 is the most potent and activates rapidly after heat shock, whereas HSF2 is a less active transcriptional regulator [Mathew et al., 2001]. Therefore, HSF1 is considered the major factor that mediates the heat shock signal in mammalian cells [Mathew et al., 2001]. HSF3 was cloned from chicken erythrocyte cDNA library and no human homologues have been described [Nakai et al., 1995]. HSF4 was found by using chicken HSF3 as a probe to screen a human cDNA library and was found to have a molecular weight of 55 kDa [Nakai et al., 1995].

In our EMSA experiments, the HSE incubated with nuclear extracts from heat shocked cells produced several bands which altered their mobility in the presence of anti-HSF1 but not the anti-HSF2 antibody (Fig. 6B). We conclude that HSF1 is the main heat-activated transcription factor that binds to HSE under our experimental conditions (Fig. 6B). When the 26-mer fragment was used as a probe, we found three specific DNA–protein complexes with nuclear extracts from untreated Jurkat or Hela cells (Fig. 3A,B). Since Hela cells do not express either HSF3 or HSF4, the binding cannot be explained by the presence of these factors. Importantly, the molecular weight of the binding protein (96 kDa) is much larger than the reported molecular weight of HSF4, 55 kDa [Nakai and Morimoto, 1993]. In untreated cells, chicken HSF3 is present exclusively in the cytoplasm as an inactive dimer with no DNA binding ability; it is only translocated into the nucleus and converted to an active trimer upon heat stress [Nakai and Ishikawa, 2000]. Additionally, the Southwestern blot was run under denaturing conditions so that the HSF members would run as monomers precluding the formation of DNA–protein complexes. Finally, since the pattern of 26-mer complexes does not change regardless of whether nuclear extract is prepared from heat shocked or untreated cells, and anti-HSF1 or HSF2 antibodies do not affect their pattern (Fig. 6D), we conclude that the putative transcription factor bound to the 26-mer DNA is not HSF1 or HSF2. It is intriguing that there is a protein–DNA complex formed when the HSE oligonucleotide is incubated with untreated Jurkat nuclear extracts that can be competed away by a 100-fold excess of cold 26-mer oligonucleotide (Fig. 6B). Furthermore, there is a reciprocal competition when the 26-mer oligonucleotide is incubated with untreated Jurkat nuclear extracts and the protein–DNA complex formed can be competed away by a 100-fold excess of cold HSE oligonucleotide (Fig. 6C). Since neither SP-1 nor AP-1 oligonucleotides can compete away either protein–DNA complex, this likely represents a specific interaction. This implies that an identical or related protein(s) is specifically interacting with both sequences under basal conditions.

The human MCT-1 oncogene has been shown to be overexpressed in both lymphoid cell lines [Prosniak et al., 1998] as well as some primary tumors [Shi et al., 2003]. Many of the lymphoid cell lines with MCT-1 overexpression have no gene amplification. This suggests that transcriptional and/or post-transcriptional regulation of the MCT-1 oncogene may play a role in its increased expression in lymphoid malignancies. Therefore, it is conceivable that the 26-mer fragment or its putative interacting DNA-binding protein, LMBF described in this work

may be involved in regulation of MCT-1 expression in human lymphoid tumors.

In summary, we have demonstrated that the MCT-1 promoter does not contain a canonical TATA box, and that an Inr is responsible for basal transcription and determine the precise site of initiation. MCT-1 transcription is facilitated by two Sp1 sites located immediately upstream of the Inr. An 11 bp sequence in the promoter, ATCTTGACCCT, together with its flanking sequences is critical for maximal transcription activity of the MCT-1 promoter and has enhancer-like activity. We have also identified by Southwestern blotting a 96 kDa nuclear protein that binds to the 26-mer DNA fragment containing this 11 bp sequence. Even though the 26-mer fragment contains a HSF consensus, the 26-mer oligonucleotide binding protein (LMBF) is not a member of known HSFs. Ongoing studies in our laboratory are focused on purifying and determining the function of this putative transcription factor.

REFERENCES

Abrescia C, De Gregorio E, Frontini M, Mantovani R, Di Nocera P. 2002. A novel intragenic sequence enhances Inr-dependent transcription in human embryonic kidney 293 cells. J Biol Chem 277:19594–19599.

Aravind L, Koonin EV. 1999. Novel predicted RNA-binding domains associated with the translation machinery. J Mol Evol 48:291–302.

Blackwood EM, Kadonaga JT. 1998. Going the distance: A current view of enhancer action. Science 281:60–63.

Burke TW, Kadonaga JT. 1997. The downstream core promoter element, DPE, is conserved from *Drosophila* to humans and is recognized by TAFII60 of *Drosophila*. Genes Dev 11:3020–3031.

Dierov J, Prosniak M, Gallia G, Gartenhaus RB. 1999. Increased G1 cyclin/cdk activity in cells overexpressing the candidate oncogene, MCT-1. J Cell Biochem 74:544–550.

Gartenhaus RB, Michaels F, Hall L, Gallo RC, Reitz MS. 1991. Relative activities of HIV-1-III$_B$ and HIV-1$_{BaL}$ LTR and *Tat* in primary monocytes and lymphocytes. Aids Res Hum Retrov 7:681–688.

Goodson ML, Park-Sarge O-K, Sarge KD. 1995. Tissue-dependent expression of heat shock factor 2 isoforms with distinct transcriptional activities. Mol Cell Biol 15:5288–5293.

Herbert GB, Shi B, Gartenhaus RB. 2001. Expression and stabilization of the MCT-1 protein by DNA damaging agents. Oncogene 20:6777–6783.

Kaufmann J, Smale ST. 1994. Direct recognition of initiator elements by a component of the transcription factor IID complex. Genes Dev 8:821–829.

Mathew A, Mathur SK, Jolly C, Fox SG, Kim S, Morimoto RI. 2001. Stress-specific activation and repression of heat shock factor 1 and 2. Mol Cell Biol 21:7163–7171.

Means AL, Farnham PJ. 1990. Transcription initiation from the dihydrofolate reductase promoter is positioned by HIP1 binding at the initiation site. Mol Cell Biol 10:652–661.

Monni O, Joensuu H, Franssila K, Knuutila S. 1998. DNA copy number changes in diffuse large B-cell lymphoma—comparative genomic hybridization study. Blood 87:5269–5278.

Nakai A, Ishikawa T. 2000. A nuclear localization signal is essential for stress-induced dimer-to-trimer transition of heat shock transcription factor 3. J Biol Chem 275: 34665–34671.

Nakai A, Morimoto RI. 1993. Characterization of a novel chicken heat shock transcription factor, heat shock factor 3, suggests a new regulatory pathway. Mol Cell Biol 13:1983–1997.

Nakai A, Kawazoe Y, Tanabe M, Nagata K, Morimoto RI. 1995. The DNA-binding properties of two heat shock factors, HSF1 and HSF3, are induced in the avian erythroblast cell line HD6. Mol Cell Biol 15:5268–5278.

Nakai A, Tanabe M, Kawazoe Y, Inazawa J, Morimoto RI, Nagata K. 1997. HSF4, a new member of the human heat shock factor family, which lacks properties of transcriptional activator. Mol Cell Biol 17:469–481.

Prosniak M, Dierov J, Okami K, Tilton B, Jameson B, Sawaya E, Gartenhaus RB. 1998. A novel candidate oncogene, MCT-1, is involved in cell cycle progression. Cancer Res 58:4233–4237.

Rabindran SK, Giorgi G, Clos J, Wu C. 1991. Molecular cloning and expression of a human heat shock factor, HSF1. Proc Natl Acad Sci USA 88:6906–6910.

Schuetz TJ, Gallo GJ, Sheldon L, Tempst P, Kingston RE. 1999. Isolation of a cDNA for HSF2: Evidence for two heat shock factor genes in human. Proc Natl Acad Sci USA 88:6911–6915.

Sheikh MS, Fernandez-Salas E, Yu M, Hussain A, Dinman JD, Peltz SW, Huang Y, Fronace AJ, Jr. 1999. Cloning and characterization of a human genotoxic and endoplasmic reticulum stress-inducible cDNA that encodes translation initiation factor 1 (eIF1 $^{A121/SUI1}$). J Biol Chem 274:16487–16493.

Shi B, Hsu HL, Evens AM, Gordon LI, Gartenhaus RB. 2003. MCT-1 oncogene expression in B- and T-lymphoid malignancies. 102:297–302.

Smale ST. 1997. Transcription initiation from TATA-less promoters within eukaryotic protein-coding genes. Biochimica et Biophysica Acta 1351:73–88.

Strachan T, Read AP. 1999. Human gene expression. In: Strachan T, Read AP, editors. Human molecular genetics. New York: Wiley-Liss. pp 172–176.

Werner CA, Dohner H, Joos S, Trumper LH, Baudis M, Barth TF, Ott G, Lichter P, Bentz M. 1997. High-level DNA amplifications are common genetic aberrations in B-cell neoplasms. Am J Pathol 15:355–342.

Yan DH, Hung MC. 1991. Identification and characterization of a novel enhancer for the rat *neu* promoter. Mol Cell Biol 11:1875–1882.