Gfi-1 Encodes a Nuclear Zinc Finger Protein That Binds DNA and Functions as a Transcriptional Repressor

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The *Gfi-1* proto-oncogene encodes a zinc finger protein with six C₂H₂-type, C-terminal zinc finger motifs and is activated by provirus integration in T-cell lymphoma lines selected for interleukin-2 independence in culture and in primary retrovirus-induced thymomas. *Gfi-1* expression in adult animals is restricted to the thymus, spleen, and testis and is enhanced in mitogen-stimulated splenocytes. In this report, we show that *Gfi-1* is a 55-kDa nuclear protein that binds DNA in a sequence-specific manner. The *Gfi-1* binding site, TAAATCAC(A/T)GCA, was defined via random oligonucleotide selection utilizing a bacterially expressed glutathione *S*-transferase-*Gfi-1* fusion protein. Binding to this site was confirmed by electrophoretic mobility shift assays and DNase I footprinting. Methylation interference analysis and electrophoretic mobility shift assays with mutant oligonucleotides defined the relative importance of specific bases at the consensus binding site. Deletion of individual zinc fingers demonstrated that only zinc fingers 3, 4, and 5 are required for sequence-specific DNA binding. Potential *Gfi-1* binding sites were detected in a large number of eukaryotic promoter-enhancers, including the enhancers of several proto-oncogenes and cytokine genes and the enhancer of the human cytomegalovirus (HCMV) major immediate-early promoter, which contains two such sites. HCMV major immediate-early-chloramphenicol acetyltransferase reporter constructs, transfected into NIH 3T3 fibroblasts, were repressed by *Gfi-1*, and the repression was abrogated by mutation of critical residues in the two *Gfi-1* binding sites. These results suggest that *Gfi-1* may play a role in HCMV biology and may contribute to oncogenesis and T-cell activation by repressing the expression of genes that inhibit these processes.

Stimulation of normal T cells by a variety of mitogenic signals induces the expression of interleukin-2 (IL-2) and IL-2 receptor alpha. Expression of these molecules takes place within 2 to 5 h of the start of the stimulation and leads to the expression of additional cytokines which induce the complex interplay of growth and differentiation events characteristic of the process of T-cell activation (reviewed in reference 10). To study the genetic regulation of the early steps of this process, we adopted an insertional-mutagenesis-based genetic strategy which was designed to identify genes contributing to the progression of IL-2-dependent T-cell lymphoma lines to IL-2-independent growth. We reasoned that genes involved in the phenotypic progression of these cell lines to IL-2 independence would play a role either in the induction of IL-2 expression or in IL-2 signaling (16).

The growth factor independence 1 (*Gfi-1*) gene cloned by using this strategy encodes a zinc finger protein with six C₂H₂-type C-terminal zinc finger motifs. *Gfi-1* expression in adult animals is restricted to the thymus, spleen, and testis. In mitogen-stimulated spleen cells, its expression begins to rise approximately 12 h after the start of the stimulation, suggesting that it is unlikely to be involved in the induction of IL-2 expression during T-cell activation. In agreement with this, *Gfi-1* failed to induce the expression of IL-2 when introduced into the IL-2-dependent cell line 2780d, which gave rise to the IL-2-independent 2780i-5 cells from which it was cloned (16).

Gfi-1 not only is involved in the selection for IL-2 independence but also contributes to the induction and progression of

a variety of rodent hematopoietic neoplasms. Thus, T-cell lymphomas arising in mice inoculated with the mouse AIDS virus frequently carry provirus insertions in *Gfi-1* (34). Provirus insertions in *Gfi-1* and a locus (*Evi-5*) located approximately 18 kb 5' of *Gfi-1* were also detected in hematopoietic neoplasms arising spontaneously in AKXD mice (28). Finally, T-cell lymphomas arising in Moloney murine leukemia virus-inoculated Eμ-*pim-1* or Eμ-*myc* transgenic mice carry provirus insertions either in a locus called *pal-1*, which is identical to *Gfi-1*, or in *Bmi-1* (5). These last findings suggest that *Gfi-1* may cooperate with *c-myc* and *pim-1* in oncogenesis but may be functionally redundant with *Bmi-1*.

To address the role of *Gfi-1* in the selection for IL-2 independence and in oncogenesis, we first expressed the genes in IL-2-dependent cell line 2780d (16, 26). These early studies showed that *Gfi-1*-expressing 2780d cells did not grow in the absence of IL-2, but when selected for IL-2 independence, they gave rise to IL-2-independent cell lines with high frequency (16). This suggested that although *Gfi-1* may not be sufficient to promote IL-2-independent growth, it may prolong cell survival in the absence of IL-2. In this communication, we present evidence that *Gfi-1* is a nuclear protein which binds DNA in a sequence-specific manner and functions as a transcriptional repressor. DNA binding is mediated by three of the six *Gfi-1* zinc fingers. Potential *Gfi-1* binding sites were detected in the promoter-enhancers of several proto-oncogenes and cytokine genes. The major immediate-early (MIE) promoter of human cytomegalovirus (HCMV) contains at least two potential *Gfi-1* binding sites. Mutation of these sites abolished DNA binding and repression. These results suggest that *Gfi-1* may play a role in HCMV biology and may contribute to T-cell activation and oncogenesis by repressing genes whose expression inhibits these processes.

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MATERIALS AND METHODS

Bacterial fusion proteins. Expression constructs of *Gfi-1* in the pGEX vector (Pharmacia) were used to express glutathione *S*-transferase (GST)–*Gfi-1* fusion proteins in *Escherichia coli*. Different constructs were designed to express the entire *Gfi-1* protein, the zinc finger domain, and the zinc finger domain carrying deletions of individual zinc fingers. Constructs with deletions of individual zinc fingers were generated by overlap extension PCR (21). *E. coli* cells transformed and selected in ampicillin were grown to log phase and induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG; Sigma). Three hours following induction, the bacteria were centrifuged and sonicated in cold phosphate-buffered saline (PBS). The lysates were then clarified by centrifugation at $15,000 \times g$ for 10 min at 4°C and mixed with glutathione-linked agarose beads (Sigma) (1/1,000 of the volume of the induced bacterial culture). Following a 10-min incubation, the beads were washed three times in cold PBS. If the fusion protein was to be eluted, the pelleted beads were washed with 10 mM reduced glutathione (Sigma) in 50 mM Tris-HCl, pH 8 (47). The liquid was collected and concentrated in a Centrprep-30 column (Amicon). Protease inhibitors [4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF) (Calbiochem) and aprotinin (Sigma)] were added prior to freezing. Sequential dilutions of the purified proteins were electrophoresed in sodium dodecyl sulfate (SDS)–polyacrylamide gels, and their relative concentration was determined by Coomassie blue staining.

Gfi-1 DNA binding: random oligonucleotide selection. A 54-base single-stranded DNA oligonucleotide was synthesized to contain a central region of 18 random bases flanked by 18-base regions with defined sequences. One overlapping, complementary oligonucleotide was annealed and extended by AmpliTaq polymerase (Perkin Elmer) to yield a mixture of double-stranded DNA fragments containing >500 copies of each possible permutation. This mixture was incubated with GST–*Gfi-1* fusion protein bound to beads (prepared as described in the previous paragraph) in binding buffer [0.2 mg of poly(dI-dC) (Sigma) per ml; 0.2 mg of acetylated bovine serum albumin (BSA; New England Biolabs), 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5), 100 mM KCl, 0.1 mM ZnSO₄, 10 mM MgCl₂, 0.1% Nonidet P-40, 1 mM dithiothreitol (DTT), 5% glycerol] for 30 min. The beads were centrifuged and washed four times with binding buffer and then boiled for 5 min in H₂O. The oligonucleotide mixture eluted from the beads by boiling was used for PCR amplification with the same amplifying oligonucleotides (32). After four rounds of selection-amplification, the PCR products were digested with *Eco*RI and *Hind*III and cloned into pBluescript (Stratagene). Alkaline-lysis-prepared plasmid DNAs from individual *E. coli* transformants (44) were sequenced (Amersham Sequenase 2.0 kit).

Electrophoretic mobility shift assays (EMSA), DNase I footprinting, and dimethylsulfate (DMS) methylation interference. Double-stranded DNA frag-

ments of selected *Gfi-1* binding sites were produced by PCR amplification of plasmid templates with oligonucleotides end labeled with [γ -³²P]ATP and T4 polynucleotide kinase (New England Biolabs). PCR products and *Gfi-1* protein were incubated in EMSA binding buffer [4 mM Tris-HCl (pH 7.5), 80 mM NaCl, 0.5 mM ZnSO₄, 1 mM EDTA, 0.5 mM DTT, 1 μ g of poly(dI-dC), 1 μ g of poly(dA-dT) (Sigma), 5% glycerol] at 22°C for 30 min. Samples were electrophoresed for ~3 h at 100 V on prerun, nondenaturing polyacrylamide gels (6% [29:1] acryl-bisacrylamide, 0.25 $\times$ TBE, 5% glycerol) (18). To determine the relative importance of individual zinc fingers for binding to the *Gfi-1* consensus, we generated a series of zinc finger deletion mutants in which all of the zinc fingers were deleted one at a time (Δ ZnF1 to Δ ZnF6). *E. coli* cells transformed with these constructs were induced, and proteins were purified and quantitated as described in the paragraph on bacterial fusion proteins. EMSA were done with equal amounts of wild-type and mutant proteins. To determine the relative importance of specific bases for *Gfi-1* binding, we also did EMSA with 24-bp-long double-stranded mutant oligonucleotides. These oligonucleotides were synthesized in vitro and end labeled with [γ -³²P]ATP and T4 polynucleotide kinase.

DNase I footprinting was done by a modified form of a previously described technique (29). PCR products of selected *Gfi-1* binding sites, 5' labeled with ³²P, and recombinant *Gfi-1* protein were mixed in 100 μ l of DNase I buffer [10 mM Tris-HCl (pH 7.8), 10 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.5 mM ZnSO₄, 0.5 mM EDTA, 0.1 mM DTT, 0.1 mg of acetylated BSA per ml, 2 μ g of poly(dI-dC), 2% polyvinyl alcohol (molecular mass, 30 to 70 kDa; Sigma), 5% glycerol] and incubated at 4°C for 30 min. The DNA was then digested for 2 min at 4°C by adding ~2 μ g of DNase I (Sigma). The reaction was stopped by adding a 1:1 mixture of phenol-chloroform. The partially digested DNA was ethanol precipitated in the presence of 10 μ g of glycogen (Boehringer Mannheim) and electrophoresed in a standard 8% polyacrylamide–8 M urea gel. The fractionated DNA fragments were visualized by autoradiography and phosphor-imager (Fuji) for quantitation.

DMS methylation interference assays were done by using a modified standard protocol (3). PCR-amplified *Gfi-1* binding site oligonucleotides, 5' labeled with ³²P, were mixed with 1 μ l of DMS in 100 μ l of DMS buffer [50 mM sodium cacodylate, 1 mM EDTA, 4 μ g of poly(dI-dC)] at 22°C for 2 min. The methylation reaction was stopped with 25 μ l of DMS stop buffer (0.86 M Tris-HCl [pH 7.5], 1.5 M sodium acetate, 1 M 2-mercaptoethanol). The DNA was then ethanol precipitated, washed twice, and resuspended in TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). The partially methylated DNA was incubated with and without *Gfi-1* protein and subsequently electrophoresed in a nondenaturing polyacrylamide gel. Input DNA (not incubated with *Gfi-1*) and shifted DNA (*Gfi-1* bound) were extracted from crushed gel fragments by incubation in acrylamide

FIG. 1. *Gfi-1* expression and subcellular localization. (A) Western blot analysis of whole-cell lysates of NIH 3T3 fibroblasts, untransfected or transfected with an HA–*Gfi-1* expression construct. The HA–*Gfi-1* fusion protein was detected with anti-HA monoclonal antibody 12CA5. (B) Immunofluorescence of fibroblasts from panel A, costained with anti-HA antibody (Ab) and DAPI, which stains nuclear DNA. FITC, fluorescein isothiocyanate.

FIG. 4. Relative binding affinities of selected sites. (A) Autoradiogram of an EMSA done with equimolar amounts of recombinant GST-Gfi-1 protein and selected oligonucleotides with increasing homologies to the Gfi-1 binding consensus. The percent homology of each oligonucleotide to the calculated consensus binding site is shown. Oligonucleotides were 5' end labeled with [γ - 32 P]ATP and polynucleotide kinase. (B) Graphic representation of the data in panel A quantitated by phospho-imager analysis. (C) Histogram showing the frequency of oligonucleotides with increasing similarity to the Gfi-1 binding consensus.

amounts of lysates were mixed at 22°C with a coenzyme A reaction mixture (80 mM Tris-HCl [pH 7.8], 360 μ g of aqueous chloramphenicol per μ l, 67 μ M acetyl coenzyme A, 3 μ Ci of [3 H]acetyl coenzyme A) in a scintillation vial. Scintillation fluid (Econofluor; Dupont) was gently overlaid to fill the vial (37). 3 H counts were measured at successive time points in a scintillation counter (Beckman LS 6000IC). The data presented here are averages of six independent transfections. Similar data were obtained in three separate experiments.

Northern (RNA) blotting. Cells of the myelomonocytic cell line HL60 (9) were grown in RPMI 1640 supplemented with 10% fetal bovine serum. Approximately 10^8 cells were seeded at a concentration of 5×10^5 cells per ml in medium containing 50 nM phorbol 12-myristate 13-acetate (PMA). Cell aliquots were lysed at 0, 0.5, 1, 1.5, 4, 8, 26, and 72 h, and the lysates were processed to isolate total cell RNA (7). Equal amounts of total RNA (20 μ g) from cells lysed at all of the time points were electrophoresed in a formaldehyde agarose gel. Following transfer to a Hybond N nylon membrane (Amersham) (45), the RNA was hybridized to a *Gfi-1* probe and a ribosomal protein L32 gene probe as previously described (4, 16).

RESULTS

Gfi-1 encodes a 55-kDa nuclear protein. Sequence analysis of full-length cDNA clones revealed that *Gfi-1* encodes a zinc finger protein with six C_2H_2 -type tandem zinc finger motifs located at the C-terminal half of the protein. This suggested that Gfi-1 may function as a transcriptional regulator. A prerequisite for the predicted role of Gfi-1 in transcription is that

the Gfi-1 protein be localized in the nucleus. To determine the subcellular localization of Gfi-1, we transfected NIH 3T3 cells with a pCMV5/*Gfi-1* expression construct tagged at its N terminus with a 12-amino-acid HA epitope tag. Western blots of total cell lysates from untransfected and transfected cells probed with HA tag monoclonal antibody 12CA5 revealed that *Gfi-1* encodes a 55-kDa protein (Fig. 1A). Immunofluorescence staining of transfected and untransfected cells with the HA epitope tag antibody demonstrated that Gfi-1 is a nuclear protein (Fig. 1B).

Proof that the Gfi-1 protein binds DNA in a sequence-specific manner and oligonucleotide selection. The predicted Gfi-1 molecular structure and subcellular localization suggest that Gfi-1 is a sequence-specific DNA binding protein. To examine this hypothesis and determine the putative Gfi-1 binding site, we used the random oligonucleotide binding-selection strategy described in Materials and Methods. The library of oligonucleotides selected by four rounds of Gfi-1 binding was cloned in pBluescript, and 96 independent clones were sequenced. Of the sequenced clones, 54 (56%) contained one AATC motif, 35 (36%) contained two AATC motifs, and 7 (7%) contained none. Alignment of the sequences of the 54 clones that contained only one AATC with this motif (Fig. 2) allowed us to determine the Gfi-1 DNA binding consensus sequence [(TAAATCAC(A/T)GCA)] (Fig. 3). To confirm the consensus binding site, we repeated the Gfi-1 binding-oligonucleotide selection experiment by starting with oligonucleotides containing an AATC motif flanked by 15-bp random DNA sequences and 18-bp regions with defined sequences. The library of selected oligonucleotides obtained after three rounds of Gfi-1 binding was cloned in pBluescript. Sequencing of 24 clones confirmed the consensus binding site (AAATCACNG C).

Relative binding affinity of Gfi-1 for cloned Gfi-1 binding sites. The homology of selected Gfi-1 binding sites to the consensus ranged from 65 to 98%. This raised the question of whether the relative binding affinity of Gfi-1 for these sites correlates with their homology with the consensus sequence. To address this question, six selected oligonucleotides whose homology to the consensus binding site ranged from 65 to 98% were used for Gfi-1 binding assays and EMSA. The Gfi-1 protein used in these experiments was expressed as a GST fusion protein in *E. coli*. The results in Fig. 4A and B show that, indeed, the relative binding affinity of Gfi-1 for individual selected sites is proportional to their homology to the consensus. Figure 4C shows a histogram depicting the frequency of selected oligonucleotides with different degrees of divergence from the consensus.

Footprint analysis of the consensus binding site. To define the DNA region to which Gfi-1 binds, oligonucleotides representing four selected Gfi-1 binding sites with 72, 85, 97, and 98% homology to the consensus were incubated with a bacterially expressed GST-Gfi-1 fusion protein. The DNA-protein complexes were digested partially with DNase I and electrophoresed in polyacrylamide-urea sequencing gels. The protected DNA region in each oligonucleotide was assessed by autoradiography. Footprints were obtained only with oligonucleotides with 97 and 98% homology to the consensus. The results showed a 21-bp footprint extending eight bases 5' and nine bases 3' of the AATC motif in both cases (Fig. 5A).

Methylation interference assays of Gfi-1 binding to the consensus binding site. To define the bases in the Gfi-1 binding site that are in contact with the protein, we carried out DMS methylation interference assays with PCR-amplified Gfi-1 binding site oligonucleotides and a bacterially expressed GST-Gfi-1 fusion protein. The results (Fig. 5B) showed that nine

FIG. 5. Importance of specific bases for Gfi-1-DNA binding. (A) Autoradiogram of DNase I footprint analysis with recombinant GST-Gfi-1 protein and a near-consensus (97%) selected oligonucleotide, R21. The AATC core and its complement are in boldface. (B) Autoradiogram of DMS methylation interference analysis with recombinant GST-Gfi-1 protein and oligonucleotide R21 singly end labeled on either DNA strand. Circles show the degree of methylation interference at a given base. Open circles indicate <20% interference; one-fourth, one-half, and three-fourths shaded circles indicate interference between 20 and 40%, 40 and 60%, and 60 and 80%, respectively. Fully shaded circles indicate >80% interference with Gfi-1 binding. A question mark indicates insufficient data. (C) Combined data from panels A and B illustrated on a double-stranded representation of oligonucleotide R21. The solid line indicates the extent of the DNase I footprint. The circles are as described for panel B. (D1 to D3) Mutant binding site oligonucleotides. (D1) Synthesized oligonucleotides with point mutations are listed below wild-type oligonucleotide R21. (D2) Autoradiogram of EMSA performed with equimolar amounts of recombinant GST-Gfi-1 protein and end-labeled mutant oligonucleotides. (D3) Graph of the results of the experiment in panel D2 quantitated by phospho-imager. (E) Binding of Gfi-1 in COS-1 cell nuclear extracts to R21. An autoradiogram of EMSA with nuclear extracts from COS-1 cells transiently transfected with either the pCMV5 vector or a pCMV5Gfi-1 expression construct is shown. Probes were synthetic oligonucleotide R21 and mutant R21A.

individual bases, when methylated, interfered with Gfi-1 binding. Specifically, the methylation of guanines on the complementary strand at positions 6 and 8 greatly impaired protein-DNA binding, while methylation of adenines within and around the AATC core and guanines distal to the core had a moderate effect. The footprint and methylation interference data are summarized in Fig. 5C.

Mutations in the Gfi-1 binding site abolish Gfi-1 binding. To confirm the significance of individual bases for Gfi-1 binding, we synthesized the wild type and five mutants of one of the selected Gfi-1 binding oligonucleotides (R21) (Fig. 5D1). By using EMSA with recombinant Gfi-1 as the indicator for Gfi-1 binding to these oligonucleotides, we showed that mutations within the AATC motif greatly decreased or abolished binding while mutations outside this motif had little effect (Fig. 5D2 and 3). The R21A mutant was still able to bind Gfi-1, although at 5% of the efficiency of R21. This shows that, in agreement with the methylation interference data, a mutation at position 3 prevents binding less efficiently than a mutation at position 4.

However, the effect of other mutations could not have been predicted by the methylation interference results. Thus, a mutation at position 6 (oligonucleotide R21C) completely ablated binding while a mutation at position 8 (oligonucleotide R21D) had little effect although the residues occupying both positions appeared to be equally important on the basis of methylation interference assays.

EMSA were also done with the wild-type R21 and mutant R21A oligonucleotides and Gfi-1 protein derived from nuclear extracts of transfected COS-1 cells. The results (Fig. 5E) showed that Gfi-1 expressed in COS-1 cells bound the wild-type R21 but not the mutant R21A oligonucleotide (94 and 5% shifted, respectively), which correlates with the EMSA results obtained with bacterially expressed Gfi-1. Gfi-1 protein expression in transfected, but not in nontransfected, cells was documented by Western blotting (data not shown).

Gfi-1 zinc fingers 3, 4, and 5 are required whereas zinc fingers 1, 2, and 6 are dispensable for DNA binding. To define the portion of the Gfi-1 zinc finger domain required for DNA

FIG. 6. Determination that only zinc fingers 3, 4, and 5 are required for DNA binding by deletion of individual zinc finger motifs. (A) GST fusion proteins, each lacking one zinc finger motif. Missing fingers are represented by gaps. (B) Autoradiogram of EMSA performed with equimolar amounts of end-labeled oligonucleotide R21 and different recombinant GST-Gfi-1 zinc finger deletion mutants. (C) Graph of the results in panel B quantitated by phosphor-imager analysis.

binding, we examined the binding of bacterially expressed GST fusions of the Gfi-1 zinc finger region from which individual zinc fingers had been deleted (Fig. 6A) to selected oligonucleotide R21. Mutant proteins expressed in *E. coli* were as stable as the wild-type protein, as determined by the approximate amount of protein induced in each case and by the lack of detectable degradation products. Binding was scored by EMSA. The results in Fig. 6B reveal that zinc fingers 3, 4, and 5 are required whereas zinc fingers 1, 2, and 6 are dispensable for DNA binding. Quantitation of these data (Fig. 6C) reveals that the finger 3 deletion construct is minimally capable of binding the R21 site (3%), in contrast to the constructs with deletions of fingers 4 and 5, which demonstrated no detectable binding. Therefore, it is possible that zinc fingers 4 and 5 play the major role in sequence-specific DNA binding, with finger 3

contributing to a somewhat lesser extent. Identical results were obtained in three separate experiments.

Many eukaryotic promoters contain potential Gfi-1 binding sites. A computer database search with the Gfi-1 binding consensus revealed potential Gfi-1 binding sites in the promoter region of many genes (Eukaryotic Promoter Database [6]). Table 1 presents a list of mammalian DNA polymerase II promoters containing potential binding sites whose sequences are $\geq 79\%$ identical to that of the consensus. The list includes several proto-oncogenes and cytokine genes. Subsequently, we examined whether the Gfi-1 binding site is consistently near or overlaps with the binding site(s) of another factor(s) (Transcription Factors Database [15]). Such an association could be interpreted to suggest that Gfi-1 binds DNA in concert with another factor(s). Alternatively, it competes with another factor(s) for DNA binding. The results of this analysis revealed that $\sim 20\%$ of the putative Gfi-1 binding sites contain GAAATC, the binding site for histone H4 gene regulatory factor H4TF-1 (11).

Gfi-1 is a transcriptional repressor that downregulates the expression of the MIE promoter of HCMV. We wanted to assess the transcriptional regulatory function of Gfi-1 in promoters with Gfi-1 binding sites and used a pCMV5/ β -galactosidase expression construct to monitor transfection efficiency. To our surprise, the expression of β -galactosidase in pCMV5/ β -galactosidase-transfected NIH 3T3 cells was downregulated in a Gfi-1-dependent manner (data not shown). To determine the specificity of this effect, we examined whether the MIE promoter of HCMV in the pCMV5 vector contains Gfi-1 binding sites. This search revealed two putative binding sites localized within two of the four 18-bp repeats of this promoter (54). The two sites were 79 and 80% homologous to the Gfi-1 binding site consensus and were placed at the bases and the apexes of proposed stem-loop structures that may play a role in promoter function (54). The generation of the Gfi-1 binding sites within the two 18-bp repeats (Fig. 7A and B) was due to divergence of these repeats from the repeat consensus. To confirm and quantitate the Gfi-1-mediated repression of this promoter, HCMV MIE-CAT reporter constructs were cotransfected transiently with a Gfi-1 expression construct into NIH 3T3 cells. In addition to the wild type, two mutant reporter constructs (A and B in Fig. 7C) were also cotransfected with Gfi-1. In mutant A, the AATC cores of both putative Gfi-1 binding sites were changed into ACTC, while in mutant B, the AATC cores of the two sites were changed into AACT and AAGT, respectively. The mutant B base changes were designed so that the altered 18-bp repeats would match the repeat consensus. On the basis of the data presented in Fig. 5D1 to 3, Gfi-1 would not be expected to bind the mutant sites. Cotransfection of Gfi-1 and the wild-type HCMV MIE promoter-CAT reporter construct downregulated the activity of the promoter more than fourfold. Promoter mutations that abrogated Gfi-1 binding (Fig. 5D1 to 3) also abrogated Gfi-1-mediated repression (Fig. 7D).

The repression of the MIE promoter by Gfi-1 may be relevant to HCMV biology. Stimulation of HL60 myelomonocytic cells with PMA induces differentiation along the macrophage lineage (43), and HCMV MIE promoter constructs transfected into HL60 cells are active only in cells induced to differentiate (49, 53). Here we showed that PMA-induced differentiation is also associated with the downregulation of Gfi-1 (Fig. 8). These results are consistent with the hypothesis that expression of Gfi-1 in cells of the myelomonocytic lineage is associated with resistance to HCMV infection and, perhaps, prevention of HCMV reactivation from latency.

TABLE 1. Identification of promoters with potential Gfi-1 binding sites^a

Promoter	Source	% Identity with consensus sequence	Sequence
			TAAATCAC ₂ GCA ^b
IL-1 α	Human	80	CAAATCAATAAC
IL-1 β	Human	86	TAAATCTGTGTG
	Mouse	80	GAAATCAGTTAA
IL-4	Human	87	GAAATCAGACCA
	Mouse	87	GAAATCAGTTAA
IL-5	Human	89	TCAATCACTGTC
		85	AAAATCCCTGTT
		82	AAAATCAGAAAA
IL-6	Human	85	TAAATCTTTGTT
IFN- α	Human	86	CAAATCTGTGTT
		84	AAAATCTAAGTT
	Mouse	91	TAAATCAAAGTT
IFN- γ	Human	79	GAAATCAGTAGT
IGF-II	Rat	88	AAAATCTGAGCT
		87	CAAATCAGACCC
		84	CAAATCAGACAA
		80	AAAATCTTAGGC
		80	TAAATCCTGGGT
	Human	86	TTAATCACGGTT
		84	CAAATCCGAGTT
CSF-1	Human	89	CAAATCTTAGCA
		79	GAAATCACCTTG
	Mouse	89	CAAATCTTAGCA
		79	GAAATCACCTTG
G-CSF	Human	79	TAAATCCTGGGA
	Mouse	79	TAAATCCTGGGA
c-sis	Rabbit	84	GAAATCAGGCCA
TNF- β	Human	83	CAAATCATACTT
	Rabbit	92	CAAATCAGGGCT
TNF- α	Human	85	CAAATCCCCGCC
		80	CAAATCAGTCAG
	Mouse	82	CTAATCATTGTC
	Rabbit	86	GAAATCAGAGGG
		81	CAAATCCGGGTC
	Hamster	86	GAAATCAGAGAG
c-mos	Mouse	90	TAAATCACTCCC
c-abl	Mouse	89	TTAATCACAGTC
c-erbB2	Human	88	GGAATCACAGGA
c-myc	Human	90	TAAATCATCGCA
N-myc	Human	86	AAAATCAGGGGA
c-N-ras	Human	85	GAAATCAGACCC
		81	AAAATCAGTAAA
	Mouse	84	GAAATCAGGCCA
		81	AAAATCAGTAAA
CD8	Mouse	90	CAAATCTCAGTT

Continued

TABLE 1—Continued

Promoter	Source	% Identity with consensus sequence	Sequence
Thy-1	Mouse	88	CCAATCACAGGA
Histone H1A	Human	93	AAAATCAAAGCA
LTR	HIV	82	CCAATCAGGGAA
MIE	HCMV	80	AAAATCAACGGG
		79	GAAATCCCCGTG
IE gpUS3	HCMV	87	GAAATCACCGTG
		87	GAAATCCCAGTA
Early 2.2 kb	HCMV	83	CTAATCACGGAC
Early 2.7 kb	HCMV	84	AAAATCAGTCCG
UL36	HCMV	80	GAAATCGCGGGC
pp65	HCMV	81	CAAATCCACGCT
		79	AAAATCGGTGTT

^a This list includes selected mammalian promoters retrieved by a computer search of the Eukaryotic Promoter Database with the Gfi-1 consensus, TAAAT CAC(A/T)GCA. All of the listed potential binding sites have scored $\geq 79\%$ homology to the consensus. This cutoff represents the value for the HCMV MIE promoter binding sites which we have shown to be functional (Fig. 7). IFN, interferon; IGF, insulin-like growth factor; CSF, colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; TNF, tumor necrosis factor; LTR, long terminal repeat; IE, immediate-early; HIV, human immunodeficiency virus.

^b Gfi-1 consensus sequence.

DISCUSSION

The data in this report show that Gfi-1 is a 55-kDa nuclear protein which binds DNA in a sequence-specific manner. Gfi-1 binding to DNA in vitro generates a 21-bp footprint which extends over the 12-bp binding site, TAAATCAC(A/T)GCA. DMS methylation interference analysis identified nine bases that closely interact with the protein. Point mutations of the binding site diminished or abrogated Gfi-1 binding and confirmed the specificity of the site. Although methylation interference suggested that Gfi-1 binding depends on protein-DNA contacts both within and outside the AATC core, EMSA with oligonucleotides of the wild-type or mutant binding sites showed that only residues within the AATC core are critical.

Given that each zinc finger is expected to bind three or four bases (13, 23, 35, 50), the 12-bp size of the Gfi-1 binding site implies that only three or four of the six Gfi-1 zinc fingers are needed for sequence-specific DNA binding. This prediction was confirmed by our experimental data. Deletions of individual zinc fingers demonstrated that fingers 3, 4, and 5 are necessary for DNA binding, whereas fingers 1, 2, and 6 are dispensable.

On the basis of the known crystal structures of zinc finger protein-DNA complexes, Suzuki et al. (50, 51) have developed stereochemical rules for predicting the DNA contacts of individual zinc fingers. These rules, when applied to Gfi-1, predict a binding site with the sequence (G/t)C(G/t)NAA(G/t)CANN(G/t)ANG(T/C)XN (Fig. 9A). The core of this site, AAGCANNNG, was present in six of the selected oligonucleotides and is nearly identical to the selected core consensus, AATCANNNG. The portion of the Gfi-1 protein predicted to bind the AAGCANNNG core contains zinc fingers 3, 4, and 5, the same fingers experimentally shown to be necessary for binding. On the basis of these contact predictions, the DMS methylation

FIG. 7. The HCMV MIE promoter is specifically repressed by Gfi-1. (A) Nucleotide sequence of the HCMV MIE promoter. The two putative Gfi-1 binding sites are boxed. The four 18-bp repeats are underlined. CAAT and TATA boxes are thinly underlined. The major transcription start site is designated +1. (B) Nucleotide sequences of the two potential Gfi-1 binding sites compared with the Gfi-1 binding consensus. (C) Comparison of the nucleotide sequences of mutant Gfi-1 binding sites in HCMV (CMV) MIE promoter mutants A and B with the wild-type sites. Bases targeted for mutation in the wild-type promoter and the mutant bases in the mutant promoters are boxed. (D) Graph showing the relative CAT activities in extracts of NIH 3T3 cells transfected with wild-type and mutant HCMV MIE-CAT reporter constructs in the presence or absence of Gfi-1.

FIG. 8. Downregulation of *Gfi-1* expression in HL60 cells following PMA stimulation. Autoradiogram of a Northern blot of total HL60 cell RNA hybridized to *Gfi-1* and ribosomal protein L32 (rpL32) probes. The RNA was harvested at 0, 0.5, 1, 1.5, 4, 8, 26, and 72 h following exposure of the HL60 cells to PMA.

interference data (Fig. 5B) and existing models of zinc finger protein interaction with the major and minor grooves of B DNA (27), we constructed the three-dimensional schematic summary of Gfi-1 binding to DNA shown in Fig. 9B. In this model, finger 5 is likely to contact in the minor groove, finger 3 is likely to contact in the adjacent major groove, and finger 4 is likely to contact across the DNA strand. The AATC core which was critical for Gfi-1-DNA binding is contacted by zinc fingers 4 and 5. Deletion of these zinc fingers abolished DNA binding. Zinc fingers 1, 2, and 6 may have other functions. By extrapolation from the function of the DNA nonbinding fingers in other zinc finger proteins, zinc fingers 1, 2, and 6 may contribute to the conformation of the protein by interacting with the DNA binding fingers, or they may be involved in nonspecific protein-DNA interactions, RNA binding, or nuclear localization signaling (8, 12, 31, 38, 40, 41, 46).

Previous high-resolution structural studies indicated that C₂H₂-type zinc fingers bind exclusively in the major groove (36). It has been recognized, however, that this type of binding may be subject to topological constraint in proteins with multiple zinc fingers (36). Our methylation interference data, showing contacts with adenines at positions 2, 3, and 4 of the binding site (Fig. 5C), suggest minor-groove binding (as shown in Fig. 9B), although they do not exclude binding in the major

FIG. 9. Prediction and schematic representation of Gfi-1-DNA binding. (A) Amino acids (aa) at positions within the Gfi-1 zinc fingers predicted to contact DNA are shown in the first column. Bases predicted to be preferred for binding by these amino acids are shown in the second column. Lowercase letters in this column indicate secondary preferences. Both strands of experimentally selected oligonucleotide R21 are shown in the third and fourth columns. Thick lines indicate that the predicted base and the selected binding site match. Thin lines indicate that the secondary prediction matches the selected binding site. The AATC core and its complement (GATT) in oligonucleotide R21 are in boldface. Boxes identify bases shown, by methylation interference analysis, to be involved in protein-DNA interaction. (B) Schematic representation of the predicted binding of individual zinc fingers to the DNA double-stranded helix. Arrows show bases that may interact with Gfi-1, according to the methylation interference data. Moreover, they identify the zinc fingers which, according to the data in panel A, are likely to interact with these bases. Major and minor grooves are exaggerated to show finger 3 in the major groove, finger 5 in the minor groove, and finger 4 across the DNA strand.

groove. Therefore, Gfi-1, a protein with multiple zinc fingers, may define an alternate mode of DNA binding.

There is no experimental evidence of Gfi-1 dimerization, and the 35 selected sites with two AATC motifs showed no consistent pattern of spacing or relative orientation of these motifs. Of the 54 selected oligonucleotides with one AATC core, however, 4 contained the sequence AATCA(C/G)TGC TT, which represents the sequences AATCANNG and AAG CANNG in opposite orientations. Given that the core, AAG CANNG, is predicted to be a Gfi-1 binding motif, these four selected oligonucleotides suggest a possible cooperativity in Gfi-1 binding to DNA.

Among the genes whose promoters contain Gfi-1 binding sites are genes encoding various cytokines and other regulators of cellular proliferation and differentiation (Table 1). Although not tested directly, the induction of *Gfi-1* during T-cell activation and its upregulation by provirus insertion during oncogenesis are likely to repress the expression of such genes. *Gfi-1*, therefore, may contribute to T-cell activation, a process characterized by sequential waves of expression of cytokine and other regulatory genes (10), by regulating the timely repression of regulatory genes. Since the expression of such genes may induce growth arrest, differentiation, and apoptosis in certain cell types, their presumed repression by *Gfi-1* may also contribute to the process of oncogenesis.

One of the promoters containing Gfi-1 binding sites is the

HCMV MIE promoter, which regulates the expression of genes required for the initiation of HCMV infection and probably the reactivation of HCMV from latency (14, 24). This promoter contains two sites with 79 and 80% homology to the Gfi-1 binding site consensus. HCMV MIE promoter-CAT reporter constructs were downregulated when cotransfected with *Gfi-1* in NIH 3T3 fibroblasts. Moreover, the Gfi-1-mediated repression of the MIE promoter was abrogated when the Gfi-1 binding sites were mutated. Therefore, Gfi-1, similarly to WT1, another zinc finger protein involved in oncogenesis (30), functions as a transcriptional repressor.

The main HCMV targets *in vivo* are cells of the monocytic lineage (48, 52). *In vitro* infection studies have shown that peripheral blood mononuclear cells or human monocytic cell lines are permissive to HCMV only when induced to differentiate (22, 25, 55). Moreover, the activity of the MIE promoter of HCMV in HL60 cells is enhanced when the cells differentiate following exposure to PMA (49, 53). This report presents evidence that PMA also induces rapid downregulation of the expression of *Gfi-1*. These preliminary data suggest that *Gfi-1* and factors that regulate *Gfi-1* expression may be involved in regulating susceptibility to HCMV infection. Work addressing this hypothesis is in progress.

The results presented in this report indicate that Gfi-1 functions as a transcriptional repressor. The detection of Gfi-1 binding sites in the promoter-enhancers of several proto-on-

cogenes and cytokine genes suggests that *Gfi-1* may contribute to T-cell activation and oncogenesis by repressing the expression of such genes. Moreover, the downregulation of the MIE promoter of HCMV by *Gfi-1* suggests that *Gfi-1* may play a role in regulating permissiveness to HCMV infection.

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