Activation of the Ferritin H Enhancer, FER-1, by the Cooperative Action of Members of the AP1 and Sp1 Transcription Factor Families

Yoshiaki Tsuji, Suzy V. Torti, and Frank M. Torti
From the Departments of Cancer Biology and Biochemistry, Bowman Gray School of Medicine and Comprehensive Cancer Center of Wake Forest University, Winston-Salem, North Carolina 27157

We have previously reported that the adenovirus E1A oncogene represses the transcription of the H subunit of the mouse ferritin gene. Subsequent analyses defined FER-1, a 37-nucleotide sequence located 4.1 kilobases proximal to the start site of transcription, as the target of E1A-mediated transcriptional repression and as an enhancer of the ferritin H gene. FER-1 is composed of an AP1-like sequence followed by an element with dyad symmetry. To achieve maximal enhancer activity and transcriptional repression by E1A, both elements were essential. Using gel retardation assays, we now demonstrate that the binding complex for the AP1-like sequence of FER-1 contains JunD, FosB, and ATF1. Furthermore, JunD and FosB were able to activate FER-1 enhancer activity by transient cotransfection with ferritin H-chloramphenicol acetyltransferase reporter constructs. This augmented enhancer activity was inhibited by E1A. In addition, we have defined the minimal sequence in the dyad element of FER-1 required for protein interaction. This was determined to be a C-rich sequence to which Sp1 and Sp3 bind. Experiments with recombinant proteins indicate that members of both transcription factor families simultaneously bind FER-1. Taken together, these results elucidate molecular mechanisms involved in the transcriptional regulation of a pivotal gene in iron metabolism and provide insights into the contribution of the Sp1 family to the activation of AP1-dependent enhancers.

Ferritin is a ubiquitous and highly conserved iron binding protein that plays a role in the storage and partitioning of iron for intracellular use. It consists of two types of subunits, designated H and L. They assemble to form a 24-subunit ferritin shell in which the H:L subunit ratio reflects the tissue type and physiological status of the cell (reviewed in Ref. 1). The H and L subunits of ferritin are encoded by independent genes (2), and expression of these genes is regulated at both transcriptional and posttranscriptional levels. The level of intracellular iron is the principal posttranscriptional regulator of ferritin.

Translation of both H and L subunits of ferritin is coordinately regulated through the interaction of RNA binding proteins with the iron-responsive element in the 5′-untranslated region of the ferritin H and L mRNAs (see Ref. 3 for review). Ferritin synthesis is also regulated at transcriptional level, in which the preferential modulation of the H subunit rather than the L subunit of ferritin is frequently observed. For instance, we reported that the transcription of the H subunit of ferritin is selectively activated by the inflammatory cytokines tumor necrosis factor (4–6) or interleukin 1 (4, 7).

In addition to these cytokines, we found that the adenovirus E1A oncogene preferentially represses the transcription of the H subunit of the ferritin gene in NIH3T3 mouse fibroblasts (8). To our knowledge, E1A is the only negative modulator of transcription of ferritin H gene identified to date, and this was another example showing independent regulation of transcription in the H and L subunits of ferritin gene. We have recently identified the target sequence (termed FER-1) responsible for E1A-mediated transcriptional repression of the mouse ferritin H gene (9). FER-1 is a 37-bp composite element comprising an AP1-like sequence followed by an element with dyad symmetry, and it is located approximately 4.1 kb 5′ to the transcription initiation site of the mouse ferritin H gene. Our previous studies demonstrated that FER-1 serves as an enhancer of the mouse ferritin H gene, which augments the transcription of this gene in a position- and orientation-independent manner. We also found that within FER-1, the AP1-like sequence alone had moderate enhancer activity, the element with dyad symmetry by itself had no enhancer activity, and both elements were required for maximal enhancer activity (9). Thus, the two elements of FER-1 cooperatively activate the transcription of the ferritin H gene. In addition, E1A decreased the binding of Jun and Fos family members to the AP1-like element of FER-1 in the absence of an alteration in the binding of nuclear factors to the dyad symmetry sequence of FER-1 (9), suggesting that the main target for transcriptional repression of the ferritin H gene by E1A is the family of AP1 transcription factors in the FER-1 binding complex.

AP1 is a sequence-specific transcription factor consisting of a dimer of Jun and Fos family members (Jun family: c-Jun, JunB, and JunD; Fos family: c-Fos, FosB, fra1, and fra2). Members of the Jun family can form homo- and heterodimers and recognize a consensus DNA sequence (TGAGTCA) or related sequences. Fos family members cannot dimerize with each other but can associate with any member of the Jun family and augment transcriptional activation when compared

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2984

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The abbreviations used are: bp, base pair; kb, kilobase; CAT, chloramphenicol acetyltransferase; CRE, cAMP response element; ATF/CREB, activating transcription factor/c-AMP response element binding protein.

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† To whom correspondence should be addressed. Tel.: 910-716-0232; Fax: 910-716-0255; E-mail: ytsuji@bgsm.edu.

‡ The abbreviations used are: bp, base pair; kb, kilobase; CAT, chloramphenicol acetyltransferase; CRE, cAMP response element; ATF/CREB, activating transcription factor/c-AMP response element binding protein.
with dimers of Jun family members (reviewed in Ref. 10). In addition to Jun and Fos family members, some ATF/CREB family members can recognize the AP1 binding sequence by dimer formation with Jun or Fos family members (11). Thus, the AP1 or AP1-related nucleotide sequences can bind at least three large families of transcription factors (Jun, Fos, and ATF/CREB families), and specific dimer formation induced by different environmental stimuli may determine the specificity of genes activated through AP1 binding sequences. AP1 was originally identified as a transcription factor essential for the basal enhancer of the human metallothionein IIA and SV40 promoters (12) and was also found to be inducible by various stimuli, such as growth factors, cytokines, and UV irradiation (13, 14). The activity of AP1 is regulated by several mechanisms, including alteration in the expression of specific AP1 components, which may affect dimer formation and consequent affinity, as well as protein phosphorylation, which regulates transcriptional activity without changing the ability to bind to a target DNA sequence (reviewed in Ref. 15).

Our previous studies identified FER-1 as both an enhancer and a EIA-responsive element of the mouse ferritin H gene and suggested that members of the Jun and Fos families recognize the AP1-like element of FER-1. However, the specific proteins binding to both the element with an AP1-like sequence and the element with dyad symmetry within FER-1 have not been elucidated. Characterization of nuclear factors that act on the FER-1 enhancer element is therefore essential not only to delineating regulatory mechanisms in transcriptional control of the ferritin H gene but also to better understanding the mechanism of EIA-mediated transcriptional repression.

We report here that 1) JunD, FosB, and ATF1 are contained in the binding complex that recognizes the AP1-like sequence of FER-1; 2) JunD and FosB are able to activate FER-1 enhancer activity, and this activation retains EIA sensitivity; 3) the C-rich sequence in the dyad element of FER-1 is involved in binding of Sp1 and Sp3 transcription factors; and 4) recombinant ATF1 and Sp1 are able to bind the FER-1 element simultaneously, suggesting that transcription factors that bind to components of the FER-1 element act in concert to stimulate ferritin H transcription.

MATERIALS AND METHODS

Cell Culture—All mouse cell lines used in this study (NIH3T3 fibroblasts, F9 embryonal carcinoma cells, BNL CL.2 normal liver cells, and L929 fibroblasts) were grown in high-glucose Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Gemini Bioproducts) at 37 °C in a humidified 5% CO2 atmosphere.

Plasmids—Most of the 5′-ferritin H-CAT plasmids used in this study were described previously (9). Briefly, pBluescript II KS(−)−4.8-kb FH CAT contains 4.8 kb of the 5′ mouse ferritin H enhancer/promoter region (16) fused to the chloramphenicol acetyltransferase (CAT) gene in the pBluescript II KS(−) vector. pBluescript II KS(−)ΔStu1/EcoRV FH CAT lacks the 3.8-kb region containing FER-1 and was constructed by deletion of the Stu1 (4.13-kb)EcoRV (0.32 kb) fragment from pBluescript II KS(−)−4.8-kb FH CAT. Two copies (antisense-antisense orientation) of the 37-bp FER-1 element (5′-CCATGCAACAAGCAGTTTGGACGAGGGAACAAAAGC-3′) or three copies (antisense-antisense-sense orientation) of the 16-bp AP1-like element (5′-CCATGGCCACAAACCTTTGGA-3′) of FER-1 were inserted into the Sma1 site (0.22 kb) of pBluescript II KS(−)ΔStu1/EcoRV FH CAT to construct pBluescript II KS(−)ΔStu1/EcoRV, Smal/FER-1 CAT and pBluescript II KS(−)ΔStu1/EcoRV, Smal/ATF1-CAT, respectively. These CAT constructs were used for cotransfection assays with mouse JunD and FosB expression plasmids (Fig. 3) as well as for transfection in various mouse cell lines (Table I). Expression of JunD and FosB was under the control of the Srα promoter in the pcDEBα vector (17). An expression plasmid of mouse CREB, pHmCREB(Δ18), was kindly provided by Dr. Gunther Schutz (German Cancer Research Center, Heidelberg, Germany).

pBluescript II KS(−)−4.8-kb AP1FH CAT and pBluescript II KS(−)−4.8-kb CREFCAT were constructed by polymerase chain reaction primer-mediated mutagenesis to produce two polymerase chain reaction products of 0.79- and 0.49-kb DNA fragments by using the following two sets of primers. For pBluescript II KS(−)−4.8-kb AP1FH CAT, 5′-ATTTCACTCTACGAAAAACAAAAGC-3′ and 5′-CTTCAAAAAGTGCTGTACAGTTTGGAGG-3′ (0.79 kb inside), 5′-CCCTCCATGCTGGACTGTTTTGGGAGG-3′ (0.49 kb inside), and 5′-CTCTGGAAGGTCAGCTGAGGGG-3′ (0.49 kb inside), and 5′-CTGTGAGGTCAGCTGAGGGG-3′ (0.49 kb inside). All plasmid constructs were characterized by restriction enzyme digestion, and the nucleotide sequence of each mutated region was verified by dideoxy DNA sequencing.

Gel Retardation Assay—The oligonucleotides used for gel retardation assays were purified from 20% acrylamide, 50% urea gel, followed by elution, Sephadex G25 column chromatography, and annealing according to standard procedures (19). Synthetic double-stranded oligonucleotides were end-labeled with T4 polynucleotide kinase. DNA sequences of the oligonucleotides with deletions and mutations in the FER-1 element are shown in Fig. 4. Isolation of nuclear extracts was carried out as described previously (9). In gel supershift experiments, 5–20 μg of nuclear extracts were incubated with 2 μg of antibody to 2 or 3 h at 4 °C prior to incubation with oligonucleotide probes at room temperature for 15 min. All antibodies for gel supershift assays were purchased from Santa Cruz Biotechnology; anti-Jun family (D, sc44x), anti-ATF1 (N, sc45x), anti-JunB (N-17, sc46x), anti-JunD (329, sc74x), anti-Fox family (K25, sc253x), anti-Fos (4, sc52x), anti-FosB (102, sc48x), anti-frα (N-17, sc183x), anti-frα2 (Q-20, sc604x), anti-ATF1/CREB1 (25C10G, sc270x), anti-CREB (C-21, sc186x, and 240, sc58x), anti-ATF/CREB (C-15, sc245x), anti-Sp1 (PEP2, sc95x) and anti-Sp3 (D-20, sc644x). Recombinant human ATF1 and Sp1 were purchased from Santa Cruz Biotechnology and Promega, respectively. Binding reactions were carried out at 37 °C for 30 min in the presence of 1.25 ng of end-labeled 37-bp FER-1 probe, 100 μg of bovine serum albumin, 1 μg of poly(dI-dC) in 20 μl of total volume. Protein-DNA complexes were separated by 5% polyacrylamide gel electrophoresis at 4 °C.

DNA Transfection and CAT Assay—Transfection of test plasmids into NIH3T3 cells was performed by DEAE-dextran method (8), and F9, BNL CL.2, and L929 cells were transfected by calcium phosphate precipitation method (20). CAT assay was carried out as described previously (8), and individual spots on silica gel plates were measured by PhosphorImager analyzer (model 445Si, Molecular Dynamics).

RESULTS

FER-1 Is an Enhancer of the Ferritin H Gene in a Wide Variety of Cell Types—The FER-1 element was initially identified using NIH3T3 mouse fibroblasts (9). To determine whether the FER-1 element also participates in ferritin H transcriptional regulation in other cell types, we performed transfection experiments in the normal liver cell line BNL CL.2, the transformed cell line L929, and the teratocarcinoma cell line F9 as well. As shown in Table I, the insertion of the FER-1 element into a ferritin H-CAT construct lacking the FER-1 region increased the CAT activity in these cell lines.

| Cell line | No. of experiments | Relative CAT activity |
|-----------|--------------------|----------------------|
| BNL CL.2  | 3                  | 100 ± 448            |
| L929      | 2                  | 100 ± 283            |
| F9        | 3                  | 100 ± 614            |

FER-1 functions as a transcriptional enhancer of the ferritin H gene in a variety of mouse cell lines

15 μg of pBluescript II KS(−)ΔStu1/EcoRV FH CAT (FER-1(−)) or pBluescript II KS(−)ΔStu1/EcoRV, Smal/FER-1 CAT (FER-1(+)) were transiently transfected into BNL CL.2, L929, or F9 cells using calcium phosphate. CAT activity was quantitated by PhosphorImager analysis of thin layer chromatography plates (number of experiments is shown in the table, duplicate for each transfection), and percentage of acetylation of chloramphenicol in extracts from cells transfected with FER-1(−)/FH CAT was defined as 100%.
FIG. 1. Complexes bound to the AP1-like element of FER-1 contain JunD, FosB, and ATF1. Nuclear extracts from confluent NIH3T3 cells (5 μg for a consensus AP1 oligonucleotide probe and 20 μg for the AP1-like oligonucleotide probe) were preincubated with 2 μg of each antibody, followed by incubation with 32P-labeled consensus AP1 oligonucleotide (5'-CCATGACTCAGCACTT-3', AP1) or the AP1-like oligonucleotide of FER-1 (5'-CCATGACAAAGCACTT-3', AP1-like). Specific activity of each probe was 0.5–1 × 10^6 cpm/ng. The protein-DNA complexes were separated by 5% polyacrylamide gel electrophoresis. A, effect of Jun family antibodies: rabbit whole serum (lanes a and f), anti-Jun family (lanes b and g), anti-c-Jun (lanes c and h), anti-JunB (lanes d and i), and anti-JunD (lanes e and j); probe: the consensus AP1 (lanes a–e) and
Similar results were observed in TA1 mouse preadipocytes (data not shown). These results suggest that the FER-1 element functions as a general basal enhancer of the ferritin H gene in cell lines with widely different tissue origins.

**Complexes Bound to the AP1-like Element of FER-1 Contain JunD, FosB, and ATF1**—Our previous studies demonstrated that Jun and Fos family members are components of the binding complex in the AP1-like element of FER-1 (9). To identify the specific members of AP1 transcription factors bound to this element, we performed gel supershift experiments using antibodies specific to each member of the AP1 family. Nuclear extracts prepared from NIH3T3 cells were preincubated with either an antibody that broadly cross-reacts to the members of the Jun family or with specific antibodies against c-Jun, JunB, or JunD, followed by addition of a probe oligonucleotide with the consensus AP1 sequence (TGACTCA) or the AP1-like sequence of FER-1 (TGACGAA). As we observed previously, anti-Jun family antibody altered the mobility of the protein-DNA complexes derived from both probes (Fig. 1A, lanes b and g). We then tested the reactivity of antibodies specific to each Jun family member to the proteins bound to the consensus AP1 oligonucleotide. As shown in Fig. 1A, all antibodies induced an additional band with slower migration (lanes c–e), indicating that these three Jun family members exist in NIH3T3 cells and are able to bind to the consensus AP1 sequence. The same experiment was carried out with the AP1-like sequence of FER-1 as a probe (Fig. 1A, lanes f–j). In contrast to the results obtained with the consensus AP1 oligonucleotide, only anti-JunD antibody induced a reproducible supershift in the mobility of the binding complex (Fig. 1A, lane j), suggesting that among Jun family members, JunD is the major component of the AP1-like element binding complex.

We next tested the effect of antibodies of Fos family members in gel shift assays. As shown in Fig. 1B, addition of an antibody that was cross-reactive with each member of the Fos family caused a supershift and inhibition of original binding complexes not only to the consensus AP1 sequence but to the AP1-like sequence of FER-1 (Fig. 1B, lanes b and h), suggesting the presence of Fos family members in these protein-DNA complexes. The use of specific antibodies against individual members of the Fos family demonstrated that anti-FosB and anti-fra1 antibodies induced a supershift of the consensus AP1 sequence binding complexes (Fig. 1B, lanes d and e), whereas only anti-FosB antibody induced a supershift in the complexes bound to the AP1-like element of FER-1 (Fig. 1B, lane j). Thus, we conclude that FosB is another component of the complex that binds to the AP1-like sequence of the FER-1 element.

Because preliminary gel retardation assays had shown that an oligonucleotide with the consensus cAMP response element (CRE) (TGACGCTCA) sequence was able to compete with the AP1-like sequence of FER-1 for binding of nuclear factors (data not shown), we also assessed the binding of members of the ATF/CREB family to the AP1-like element of FER-1. As shown in Fig. 1C, anti-ATF1 antibody, which is cross-reactive to CREB1, caused a supershift in the mobility of protein complexes bound to the AP1-like element of FER-1 that was greater than that seen with the consensus AP1 element (lanes c and i). The use of antibodies specific to ATF1 and CREB1 demonstrated that anti-ATF1 antibody had the ability to alter the mobility of the AP1-like binding complexes (Fig. 1C, lane l), whereas two different antibodies to CREB1 had no effect (lanes j and k). Antibody specific to ATF1 or CREB1 did not affect the binding of factors to the consensus AP1 sequence (Fig. 1C, lanes d–f). These results suggest that ATF1 is a third component bound to the AP1-like element of FER-1. We obtained similar results when these experiments were repeated with different preparations of nuclear extracts. Thus, among the AP1 and ATF/CREB families, the AP1-like sequence of FER-1 preferentially binds JunD, FosB, and ATF1.

**The AP1-like Sequence of FER1 Is Optimal for Basal Enhancer Activity**—Selective members of the AP1 and ATF/CREB family recognize a consensus AP1 binding sequence or a consensus CREB binding sequence more strongly than related nucleotide sequences (21). This was true when the AP1-like sequence of FER-1 was changed to a consensus AP1 binding sequence, which increased total protein binding by approximately 6-fold in gel retardation assay (data not shown, but see Fig. 1). We therefore asked whether substitution of the AP1-like sequence of FER-1 for a consensus AP1 or CRE sequence is able to increase the enhancer activity of the ferritin H gene. To address this question, we introduced nucleotide changes into the AP1-like sequence (TGACGAA) of FER-1 in pBluescript II KS(−) (−4.8-kb FHCAT) or a consensus CRE site (TGACGCTCA, −4.8-kb CRE/FHCAT). These CAT constructs were cotransfected into NIH3T3 cells with pUC18 or p12SE1A into NIH3T3 cells was carried out, and autoradiographs of the thin layer chromatography plates were quantitated. Results from four independent experiments (duplicate in each experiment) and standard error are shown. The CAT activity in extracts from cells cotransfected with pBluescript II KS(−) (−4.8-kb FHCAT, −4.8-kb AP1-like, wild type) and pUC18 was defined as 100%.

**JunD and FosB Activate the FER-1 Enhancer**—We next as-
Regulation of Ferritin H by AP1/Sp1 Cooperation

A C-rich Sequence in the Dyad Element of FER-1 Represents the Minimal Element Required for Protein Binding and Transcriptional Activation—Our previous results demonstrated that a mutation in the dyad element that eliminates both dyad symmetry and the ability to bind nuclear factors decreased FER-1 enhancer activity (9). However, the key nucleotide sequences in the dyad element required for protein binding and the nature of nuclear factors binding to this element remain unknown. To address these questions, we tested FER-1 oligonucleotides containing various partial sequences of the dyad element for their ability to bind proteins in a gel retardation assay (Fig. 4). Oligonucleotide probes containing the AP1-like element alone (Fig. 4, AP1-like), the AP1-like element plus a partial dyad sequence containing a deletion of 15 nucleotides (FER-1Δ15) and the AP1-like element plus a dyad sequence containing a deletion of 9 nucleotides (FER-1Δ9) all gave rise to a similar retarded band. This represented the interaction between AP1 transcription factors and the AP1-like element, as judged by competition experiments demonstrating competition by the AP1-like oligonucleotide and absence of competition by the dyad (Fig. 4, FER-1Δ9). In contrast, reconstitution of FER-1 by the addition of 9 nucleotides conferred the ability to interact with additional proteins (see Fig. 4, FER-1, no competitor), suggesting that the complete dyad sequence or at least the 3′ portion of the dyad element including 5′-CCCTCCAAA-3′ (compare FER-1Δ9 with FER-1) is important for protein interaction. In fact, mutations in this sequence (Fig. 4, mDyad-2) consistently caused the loss of ability to bind a set of proteins.

We next tested the contribution of the 5′ portion of the dyad element to this protein interaction. For this purpose, we tested the protein binding ability of an oligonucleotide with three mutations in the 5′ portion of the dyad element. Unexpectedly, this mutant dyad oligonucleotide (Fig. 4, mDyad-2) retained the ability to bind the same set of proteins with an affinity similar to that exhibited by the nonmutated dyad element (Fig. 4, Dyad), indicating that the 5′ portion of the dyad sequence is not directly involved in protein binding. This result also suggests that the disruption of dyad symmetry per se does not necessarily affect protein binding. To further test this possibility, we made a nonsymmetrical oligonucleotide by creating additional mutations in the 3′ portion of the dyad element. These mutations (Fig. 4, mDyad-3) did not disrupt the binding of nuclear factors. These results indicate that the 3′–5′ nucleotides at the 3′ and 5′ ends of the dyad element of FER-1 are not essential for protein interaction.

We then asked whether the ability to bind proteins was necessary for activity of the dyad element of FER-1 in vivo. We introduced the same five nucleotide changes into the dyad symmetry sequence of FER-1 in the −4.8-kb FHCAT plasmid, resulting either in the loss of ability to bind proteins (−4.8-kb mDyad-1 FHCAT) or in the retention of binding ability (−4.8-kb mDyad-3 FHCAT). These CAT constructs were transiently cotransfected into NIH3T3 cells with pUC18 or p12SE1A. Despite the destruction of dyad symmetry in both constructs, a significant reduction in basal activity was only observed in the construct in which protein binding activity had been abrogated (Table II). These results suggest that the complete dyad symmetry sequence of the FER-1 element, and dyad symmetry per se are not essential for maximum enhancer activity of the ferritin H gene. Both constructs still responded to the E1A-mediated repression of promoter activity (Table II), consistent with our results suggesting that E1A represses FER-1 enhancer activity primarily through inhibition of AP1 activity (Fig. 3 and Ref. 9).

Sp1 and Sp3 Bind to the C-rich Sequence of the FER-1 Dyad Element—The middle of the dyad element has a C-rich se-
The C-rich sequence in the dyad element of FER-1 contributes to protein binding. 10 µg of nuclear extracts from confluent NIH3T3 cells were incubated with the 16-bp AP1-like oligonucleotide (AP1-like), a FER-1 oligonucleotide with a 15-nucleotide deletion in the dyad (FER-1Δ15), a FER-1 oligonucleotide with a 9-nucleotide deletion in the dyad (FER-1Δ9), the 37-bp complete FER-1 oligonucleotide (FER-1), a 22-bp dyad symmetry oligonucleotide (Dyad), an oligonucleotide with mutations in the 3' portion of the dyad (mDyad-1), an oligonucleotide with mutations in the 5' portion of the dyad (mDyad-2), or an oligonucleotide with mutations in both the 5' and 3' ends of the dyad (mDyad-3) in the presence of a 200-fold molar excess of an unlabeled oligonucleotide competitor as indicated. Mutated nucleotides in the dyad element are marked with an asterisk. The specific activities of AP1-like, FER-1Δ15, FER-1Δ9, and FER-1 probes were 0.8–1 × 10^5 cpm/ng, and the specific activities of dyad and mutated dyad probes were 2 × 10^5 cpm/ng.

Mutations in the dyad element of FER-1 affect enhancer activity of the ferritin H gene

The dyad symmetry sequence of FER-1 in −4.8-kb FHCAT was eliminated by introduction of mutations, which retained the binding ability (−4.8-kb mDyad-3) or abolished the ability of Sp1/Sp3 binding (−4.8-kb mDyad-1) as shown in Fig. 4. 15 µg of CAT construct were transiently cotransfected into NIH3T3 cells with 5 µg of pUC18 or p12SE1A using DEAE-dextran. Autoradiographs of the thin layer chromatography plates (number of experiments is shown in the table, duplicate for each transfection) were quantitated, and percentage of acetylation of chloramphenicol in extracts from cells transfected with −4.8-kb wild type FHCAT with pUC18 control was defined as 100%.

| FHCAT            | Number of experiments | relative CAT activity |
|------------------|-----------------------|----------------------|
| −4.8-kb weight   | 8                     | 100                   |
| −4.8-kb mDyad-1  | 7                     | 65 ± 6                |
| −4.8-kb mDyad-3  | 8                     | 91 ± 4                |

might play a role in protein binding. We therefore examined the possibility that Sp1 or Sp1-related factors may bind to the dyad element of FER-1. For this purpose, we performed gel retardation assays using the consensus Sp1 oligonucleotide as a probe or a competitor. As shown in Fig. 5A, the 22-bp dyad element of FER-1 and the 22-bp consensus Sp1 oligonucleotide (Promega) gave rise to a similar pattern of retarded bands, although the dyad element of FER-1 had a lower apparent affinity for nuclear proteins than the consensus Sp1 sequence (Fig. 5A, lanes a and g). In addition, the interaction between nuclear proteins and the dyad element of FER-1 was inhibited not only by the unlabeled dyad element (Fig. 5A, lanes b and c) or the complete FER-1 element (lane f) but to a further extent by the consensus Sp1 oligonucleotide in a dose-dependent fashion (lanes d and e). In the reverse experiment, the interaction between the nuclear proteins and the consensus Sp1 binding sequence was partially inhibited in a dose-dependent manner by excess unlabeled dyad oligonucleotide (Fig. 5A, lanes j and k) or the complete FER-1 element (lane l). Taken together, we conclude that the C-rich sequence in the dyad element of FER-1 contributes to the protein binding and that it binds Sp1 or Sp1-family proteins.
Regulation of Ferritin H by AP1/Sp1 Cooperation

Fig. 5. Sp1 and Sp3 bind to the C-rich sequence of the dyad element. A. 10 μg of nuclear extracts from confluent NIH3T3 cells were incubated with a 22-bp dyad symmetry oligonucleotide (Dyad, lanes a–f) or a 22-bp consensus Sp1 oligonucleotide (Promega) (Sp1, lanes g–l) in the presence of a 5-fold (lanes b and j) or 50-fold (lanes c and k) molar excess of the unlabeled 22-bp dyad oligonucleotide, a 5-fold (lanes d and h) or 50-fold (lanes e and i) molar excess of a 22-bp consensus Sp1 oligonucleotide, or a 50-fold molar excess of a 37-bp FER-1 oligonucleotide (lanes f and l). The specific activity of each probe was 2 × 10^6 cpm/ng. B, 10 μg of nuclear extracts from confluent NIH3T3 cells were incubated with a 22-bp dyad symmetry oligonucleotide (Dyad, lanes a–d), a 22-bp consensus Sp1 oligonucleotide (Promega) (Sp1, lanes e–h) or a 37-bp FER-1 oligonucleotide (FER-1, lanes i–l) after preincubation alone (lanes a, e, and i) or with 2 μg of normal rabbit serum (lanes b, f, and j), anti-Sp1 (lanes c, g, and k), or anti-Sp3 (lanes d, h, and l) antibody. The specific activity of each probe was 2 × 10^6 cpm/ng.

Recently, a number of transcription factors related to Sp1 have been discovered, some of which may repress Sp1-mediated transcriptional activation by competition for identical binding sequences (24, 25). Based on a comparison of the mobility of bands observed in our gel retardation experiments with published gel retardation assays for Sp1 family members (25–27), we speculated that Sp1 and Sp3 might bind to the dyad element of FER-1. To test this possibility, we preincubated NIH3T3 nuclear extracts with anti-Sp1 or anti-Sp3 antibody prior to addition of an oligonucleotide probe. The 22-bp dyad element of FER-1, the 22-bp consensus Sp1 (Promega), and the 37-bp FER-1 oligonucleotide were used as probes for gel retardation assays. As shown in Fig. 5B, in all three cases, anti-Sp1 antibody induced a supershift in the upper band without any effect on other two bands (lanes c, g, and k). On the other hand, anti-Sp3 antibody caused a supershift of the middle and lower bands (lanes d, h, and l). Preimmune serum (lanes b, f, and j) was without effect. The fact that the combination of anti-Sp1 and anti-Sp3 antibodies completely retarded the original bands (data not shown) indicates that the major binding proteins to the dyad element of FER-1 are Sp1 and Sp3.

Binding of Recombinant Proteins to the FER-1 Element—We have previously demonstrated that the AP1-like element and the dyad element of FER-1 cooperatively activate the transcription of the ferritin H gene (9). To determine whether this synergy is due to the interaction of binding proteins to the two elements of FER-1, we tested the ability of purified recombinant proteins to bind to the FER-1 element. A gel retardation assay was performed using recombinant ATF1 and Sp1, both of which were detected in the binding complex of FER-1 in gel supershift experiments (Figs. 1C and 5B). In support of the supershift experiments, both recombinant proteins individually bound to the FER-1 element in a dose-dependent fashion (Fig. 6, lanes a–d). When ATF1 and Sp1 proteins were mixed together in the binding reaction, both were able to recognize the FER-1 element with no apparent effect of the binding of one protein on the affinity of the other (Fig. 6, compare lanes a and c with lane e). At higher concentrations of ATF1 and Sp1, a new complex with slower migration was detected (lane f). This complex was not affected by control rabbit IgG, but it disappeared following incubation with anti-ATF1 or anti-Sp1 antibody (data not shown), suggesting the formation of ternary complex between these two recombinant proteins and the FER-1 element. These results suggest that the two elements of FER-1 have the ability to bind the corresponding transcription factors simultaneously and that one transcription factor (e.g. ATF1) does not recruit the other (e.g. Sp1) to the FER-1 element.

DISCUSSION

Ferritin H, a gene expressed in most tissues, is subject to transcriptional control in response to a variety of endogenous and exogenous stimuli, including oncogenes, cytokines, hormones, and chemical inducers of differentiation (5, 7, 28–30). The 5′ regulatory region of ferritin H reflects this complex repertoire of responses. We have identified two elements, FER-1 and FER-2, located approximately 4-kb 5′ to the transcriptional start site of ferritin H. FER-2, located at −4.7 kb,
mediates tumor necrosis factor responsiveness through binding of nuclear factor κB factors (31); FER-1, located at ≈4.1 kb, mediates basal enhancer activity through interaction with an independent set of transcription factors, which we identify here. FER-1 is also a target of E1A-mediated transcriptional repression (8). FER-1 is composed of two elements: an AP1-like sequence followed by an element with dyad symmetry (9). In the present study, we have characterized specific transcription factors that interact with these components of the FER-1 element. Binding proteins interacting with the AP1-like element of FER-1 were identified as JunD, FosB, and ATF1 by gel retardation assays using antibodies specific to AP1 and ATF/CREB family members (Fig. 1).

Through genetic modification of the FER-1 sequence, we were able to test whether this specific constellation of transcription factors was in fact required for optimal enhancer activity of FER-1. When the 16-bp AP1-like sequence in the FER-1 element containing TTAGAATA was changed to the consensus AP1 binding sequence TGACTCA, the affinity of total protein binding was increased by approximately 6-fold in a gel retardation assay (data not shown, but see Fig. 1). This was accompanied by a change in members of the AP1 and ATF/CREB transcription factors in the binding complex (Fig. 1). However, the conversion of the AP1-like sequence of FER-1 into the consensus AP1 sequence in the −4.8-kb ferritin-HCAT did not further increase CAT expression following transient transfection into NIH3T3 cells (Fig. 2). The report that cAMP increases ferritin H transcription in rat thyroid cells (29) and in rabbit smooth muscle cells (32) suggests that other ATF/CREB family members may also participate in the mouse ferritin H regulation through the FER-1 element. However, the conversion of the AP1-like sequence into a consensus CRE sequence (TGACGTCA) did not increase the FER-1 enhancer activity; rather, it caused it to decrease (Fig. 2). These results suggest that the particular array of transcription factors bound to FER-1 is optimal for transcriptional activation of the ferritin H gene.

Using functional assays, JunD and FosB were shown to activate ferritin H enhancer activity in F9 cells (Fig. 3), whereas CREB failed to do so (data not shown), suggesting that these AP1 family proteins contribute to ferritin H transcription in vivo. However, it should be noted that transfection of a c-Jun expression plasmid alone (data not shown) or c-Jun plus c-Fos expression plasmids (9) was also able to stimulate the FER-1-CAT reporter plasmid in F9 cells despite the apparent lack of binding of c-Jun or c-Fos proteins to the AP1-like sequence of FER-1 (Fig. 1). Although we do not have a precise explanation for these results, a possibility is that overexpressed c-Jun and c-Fos may be able to bind weakly to the AP1-like element of FER-1 or recruit endogenous JunD, FosB, or ATF1 to form a dimer competent to recognize the AP1-like element of FER-1, or that they may transcriptionally induce JunD, FosB, or ATF1 expression. These mechanisms may serve to confer a flexibility to the transcriptional regulation of ferritin H that may come into play in different cell types or under different physiological conditions.

Our previous studies demonstrated that the AP1-like element of FER-1 by itself had moderate enhancer activity and required a second element with dyad symmetry for maximal enhancer activity (9). Analysis of the key nucleotide sequences in the dyad element essential for protein interaction presented here demonstrated that a C-rich sequence (AGCCCAACCCCTCC) in the dyad element plays a role in binding of nuclear factors (Fig. 4). This C-rich sequence has homology to the binding site of GC box binding factors, Sp1, or Sp1-related proteins. In gel retardation assays, we found that the binding of proteins to the dyad element was in fact strongly competed by the consensus Sp1 oligonucleotide (Fig. 5A), suggesting that the C-rich sequences in the dyad element of FER-1 bind GC box binding factors, including Sp1. Among the Sp1-related transcription factors so far described (33), we identified Sp1 and Sp3 as the transcription factors associated with the dyad element of FER-1 (Fig. 5B). In addition, our results indicated that dyad symmetry per se was not important for protein binding or enhancer activity of FER-1 (Table II).

Our results provide evidence for a cooperative activation of the ferritin H enhancer by members of the AP1 and Sp1 transcription factor families. Although Sp1 by itself has the ability to activate the transcription of target genes, several lines of evidence have recently indicated that Sp1 also interacts and functionally cooperates with other transcription factors, such as nuclear factor κB (34) on composite binding elements of HIV-1 enhancer. Very recently, the cooperativity of AP1 and Sp1 in the transcriptional activation of human granulocyte-macrophage colony-stimulating factor (35), human leukocyte integrin, CD11c (36), and human P class glutathione S-transferase (37) has been reported. Our results suggest that this cooperative interaction extends to a broad range of both AP1 and Sp1 family members, including JunD and FosB, as well as Sp1/Sp3. Although the mechanisms underlying these effects remain to be clarified, it has been suggested that in the case of the CD11c promoter, Sp1 binding facilitates the binding of AP1 (36). In contrast, our gel retardation experiments indicated that the dyad element and the complete FER-1 element (the AP1-like element plus the dyad element) were equivalently effective as competitors for binding to an Sp1 element (Fig. 5A, lanes k and l). These results, as well as experiments with recombinant transcription factors (Fig. 6), suggest that the cooperative effect of AP1 and Sp1 family members in ferritin H transcriptional activation may proceed via a mechanism that does not involve recruitment of one transcription factor by the other.

The effect of Sp1 and/or Sp3 on ferritin H transcription was evident as a substantial augmentation of enhancer activity when compared with that driven by AP1 alone (Fig. 3). However, the inhibition of FER-1 enhancer activity by E1A did not appear to target the binding activity of Sp1. Thus, E1A did not alter binding of Sp1/Sp3 to FER-1 or facilitate the replacement of Sp1 family members bound to FER-1 by alternative inhibitory family members (data not shown) as seen in the repression of the fibroectin gene by E1A, in which a negative regulator (G10BP) competes for the binding of Sp1 to the G-rich sequences in the promoter region and represses transcription (38). In contrast, our previous results have demonstrated that E1A decreases binding of AP1 family members to FER-1 (9). Further experiments will be required to address whether the transcriptional inhibitory properties of E1A reside solely in its ability to target AP1 or whether modulation of the transcriptional activation properties of Sp1 and/or Sp3 or of transcriptional adaptor proteins also plays a role in the complex response of the ferritin H gene to multiple environmental and intracellular signals. The role of FER-1 in ferritin H transcriptional activation in a variety of cell types (Table I) suggests that elucidation of mechanisms of interaction of these transcription factors will provide insight into transcriptional regulation of this critical component of iron metabolism.

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