FER-1, an Enhancer of the Ferritin H Gene and a Target of E1A-Mediated Transcriptional Repression

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Ferritin, the major intracellular iron storage protein of eucaryotic cells, is regulated during inflammation and malignancy. We previously reported that transcription of the H subunit of ferritin (ferritin H) is negatively regulated by the adenovirus E1A oncogene in mouse NIH 3T3 fibroblasts (Y. Tsuji, E. Kwak, T. Saika, S. V. Torti, and F. M. Torti, J. Biol. Chem. 268:7270–7275, 1993). To elucidate the mechanism of transcriptional repression of the ferritin H gene by E1A, a series of deletions in the 5′ flanking region of the mouse ferritin H gene were constructed, fused to the chloramphenicol acetyltransferase (CAT) gene, and transiently cotransfected into NIH 3T3 cells with an E1A expression plasmid. The results indicate that the E1A-responsive region is located approximately 4.1 kb 5′ to the transcription initiation site of the ferritin H gene. Further analyses revealed that a 37-bp region, termed FER-1, is the target of E1A-mediated repression. This region also serves as an enhancer, augmenting ferritin H transcription independently of position and orientation. FER-1 was dissected into two component elements, i.e., a 22-bp dyad symmetry element and a 7-bp AP1-like sequence. Insertion of these DNA sequences into a ferritin H-CAT chimeric gene lacking an E1A-responsive region indicated that (i) the 22-bp dyad symmetry sequence by itself has no enhancer activity, (ii) the AP1-like sequence has moderate enhancer activity which is repressed by E1A, and (iii) the combination of the dyad symmetry element and the AP1-like sequence is required for maximal enhancer activity and repression by E1A. Gel retardation assays and cotransfection experiments with c-fos and c-jun expression vectors suggested that members of the Fos and Jun families bind to the AP1-like element of FER-1 and contribute to its regulation. In addition, gel retardation assays showed that E1A reduces the ability of nuclear proteins to bind to the AP1-like sequence without affecting the levels of nuclear factors that recognize the 22-bp dyad symmetry element. Taken together, these results demonstrate that FER-1 serves as both an enhancer of ferritin H transcription and a target for E1A-mediated repression.

Ferritin is the major intracellular iron storage protein in eucaryotic cells, and it plays a prominent role in maintaining intracellular iron homeostasis (44). Ferritin consists of 24 subunits of the H and L types, which associate in various ratios depending on physiological state and tissue type (2). Ferritin H and ferritin L are encoded by separate genes (22). A major regulator of ferritin synthesis is intracellular iron, which stimulates the translation of both ferritin H and ferritin L mRNAs through the interaction of an RNA-binding protein with an iron-responsive element located in the 5′ untranslated regions of ferritin mRNAs (24). In addition, ferritin is subject to transcriptional control. Several studies have demonstrated that ferritin is regulated by inflammation (38), as well as by differentiation (4, 12) and malignancy (38). We have previously demonstrated that the cytokines tumor necrosis factor alpha or interleukin-1α, both of which play a major role in the regulation of a wide variety of immune and inflammatory responses (49), selectively stimulate the transcription of the H subunit of ferritin in several cell types (45, 53). We have hypothesized that such alterations in the subunit composition of ferritin may in turn modulate the iron binding capacity of ferritin and

regulatory pools of free iron (30, 47). Others have demonstrated that additional agents which regulate ferritin, such as thyrotropin, cyclic AMP (cAMP), and differentiation, also preferentially increase the transcription of the H subunit of ferritin (12, 13, 27). Taken together, these results suggest that modulation of ferritin composition may be an important cellular response to a variety of physiological and pathophysiological stimuli.

Neoplastic cells exhibit an alteration in ferritin composition or content compared with their normal counterparts (10, 50), although the mechanism(s) by which these responses are mediated remains unknown. We have recently demonstrated that stable expression of the adenovirus early region 1A oncogene (E1A) alters the composition of ferritin protein in mouse NIH 3T3 fibroblasts via a preferential repression of ferritin H transcription (46). The E1A gene of adenovirus encodes at least five species of mRNA. Two of these, 13S and 12S mRNAs, are major species formed by the differential splicing of a single E1A transcript early in infection (43). Proteins encoded by the E1A gene of adenovirus have pleiotropic functions, including transcriptional activation, transcriptional repression, cell transformation, and immortalization (32, 43). The E1A region required for cell transformation has been mapped to two noncontiguous domains in the first exon (conserved regions 1 and 2) common to both 12S and 13S E1A products (23, 28, 41).

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TABLE 1. Oligonucleotides used in this study for construction of mutant plasmids

Mutant	Oligonucleotide(s)
−4kbAP1	5′-CTGAGTCACGGTGGAACAACAATTTTCCACAA-3′
	5′-GATCTTGTGGAAAATTGTTGTTCCACCGTGACTCAGGGCC-3′
−4kbAP1+α	5′-CGGAGCAGAATGCTGAGTCACGGTGGAACAACAATTTTCCACAA-3′
	5′-GATCTTGTGGAAAATTGTTGTTCCACCGTGACTCAGCATTCTGCTCCGGGCC-3′
−4kbAP1+β	5′-CCTCCAAAGGAGCAGAATGCTGAGTCACGGTGGAACAACAATTTTCCACAA-3′
	5′-GATCTTGTGGAAAATTGTTGTTCCACCGTGACTCAGCATTCTGCTCCTTTGGAGGGGCC-3′
−4kbAP1+γ	5′-CTTTGGAGCCCAACCCCTCCAAAGGAGCAGAATGCTGAGTCACGGTGGAACAACAATTTTCCACAA-3′
	5′-GATCTTGTGGAAAATTGTTGTTCCACCGTGACTCAGCATTCTGCTCCTTTGGAGGGGGTTGGGCTCCAAAGGGCC-3′
−4kbAP1+δ	5′-TGTGGGGCCCCATGACAAAGCACTTTTG-3′[a]
−4kbAP1+ζ	5′-TGTGGGGCCCAAAGCACTTTTGGAGCCC-3′[a]
−4kbAP1+ε	5′-TGTGGGGCCCCCTTCCAGACTGCCCCTAC-3′[a]
−4.8kb mAP1	5′-ATTAACCCTCACTAAAGGGAACAAAAGC-3′
	5′-GTGAGATCTTGTGGAAAATTGTTGTTCCACCGTGACGATGCATTCTGCTCCTTTG-3′
−4.8kb ΔDyad	5′-ATTAACCCTCACTAAAGGGAACAAAAGC-3′
	5′-AAGTGCTTTGTCATGG-3′
	5′-CGGAGCAGAATGCTGAGTCACGGTGGAACAACAATTTTCCACAA-3′
	5′-CCTGTGGAAGGTCACTG-3′
−4.8kb mDyad	5′-ACTTTTGGAGCCCAACTCGGATAAAGGA-3′[b]
	5′-TCCTTTATCCGAGTTGGGCTCCAAAAGT-3′[b]
	5′-ATTAACCCTCACTAAAGGGAACAAAAGC-3′[c]
	5′-CCTGTGGAAGGTCACTG-3′[c]

[a] 5′ PCR primer. The 3′ common primer for −4kbAP1+δ, −4kbAP1+ζ, and −4kbAP1+ε was 5′-CTGGCTGCCTGGAACAAG-3′.
[b] Inside primer.
[c] Outside primer.

These regions of the E1A proteins form complexes with a family of related cellular proteins including the p105 retino-blastoma susceptibility gene product (p105 Rb) and related proteins (19, 54, 55). The interaction of E1A with these cellular proteins alters cellular gene expression. Conserved regions 1 and 2 of E1A also play an important role in transcriptional repression of viral and cellular genes (28, 41). Although the mechanism by which E1A oncogene products repress a selected group of viral and cellular genes is not well understood, transcriptional repression of such cellular genes as the rat *neu* oncogene (57), human collagenase genes (16, 18), and the rat fibronectin gene (35) is mediated through specific enhancer sequences located in the target genes. The fact that E1A proteins do not bind directly to specific DNA sequences (15) suggests that E1A proteins may negatively regulate transcription through interaction with transcription factors.

In the present study, we have addressed the question of how adenovirus E1A represses transcription of the ferritin H gene. We have characterized the E1A-responsive element involved in the transcriptional repression of the mouse ferritin H gene by a transient chloramphenicol acetyltransferase (CAT) reporter assay and also studied nuclear factors that bind to DNA sequences within this E1A-responsive element. We report that a 37-bp region, FER-1, serves as a transcriptional enhancer and mediates responsiveness to E1A in the murine ferritin H gene.

MATERIALS AND METHODS

Cell culture. NIH 3T3 mouse fibroblasts, E1A transfectants (48), and F9 cells were cultured at 37°C in 5% CO_2 in high-glucose Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

Construction of 5′ ferritin H-CAT plasmids. The mouse ferritin H gene used in this study has been described previously (25). pBluescript II KS(−) (Stratagene) was used as a backbone plasmid for construction of the various CAT plasmids. pBluescript II KS(−) 0CAT was constructed by digestion of pSV0CAT (obtained from the American Type Culture Collection) with HindIII and BamHI, followed by isolation of the 1.6-kb CAT fragment and ligation to Hin-dIII-BamHI-digested pBluescript II KS(−). This promoterless CAT gene was not expressed when transfected into NIH 3T3 cells. For construction of pBluescript II KS(−) −4.8kbFHCAT, the 4.8-kb HindIII fragment containing the 5′

flanking region of the mouse ferritin H gene (from nucleotide −4819 to nucleotide +24 [25]) was isolated from p−5kbFHCAT (46) and ligated to HindIII-digested pBluescript II KS(−) 0CAT.

(i) 5′ deletion mutants. A series of stepwise deletion mutants of −4.8kbFHCAT (Fig. 1A) were constructed by exonuclease III-mung bean nuclease digestion. pBluescript II KS(−) −4.8kbFHCAT was digested with ApaI and ClaI and then incubated at 30 or 37°C with 4 U of exonuclease III (Stratagene) per μl. A 5-μg portion of DNA was withdrawn every 60 s, heated at 68°C for 15 min, treated with 15 U of mung bean nuclease (Stratagene) at 30°C for 30 min, extracted with phenol-chloroform and chloroform, and treated with T4 ligase.

(ii) Internal deletion mutants. The internal deletion mutants ΔKpn I, ΔXho I/Kpn I, and ΔSst I/Kpn I (Fig. 2A) were constructed as follows. ΔKpn I was made by digestion of p-5kbFHCAT with KpnI, followed by isolation and religation of the resultant 6.5-kb fragment. ΔXho I/Kpn I was created by digestion of p−5kbFHCAT with KpnI and XhoI, S1 nuclease treatment, and isolation and religation of the 5.4-kb fragment. ΔSst I/Kpn I was similarly constructed by digestion with SstI and Kpn I, S1 nuclease treatment, and blunt end ligation. The deleted ferritin H gene in these three CAT constructs was excised by using HindIII and recloned into the HindIII site of pBluescript II KS(−) 0CAT. For construction of ΔBgl II/Eco RV, pBluescript II KS(−)−4.8kbFHCAT was digested with BglII and EcoRV and blunt ends were generated by filling-in with the large fragment of DNA polymerase I. The 5.6-kb fragment was isolated and religated. ΔStu I/Eco RV was similarly constructed following partial digestion of pBluescript II KS(−)-4.8kbFHCAT with StuI.

(iii) Point mutation or deletion of the consensus AP1 site in the E1A-responsive region. pBluescript II KS(−) −4.8kb mAP1 FHCAT (Fig. 3A) was constructed by insertion of a PCR primer-mediated mutated AP1 DNA fragment into the ApaI-BglII fragment of pBluescript II KS(−) −4.8kbFHCAT. Primers used for this construction are shown in Table 1. For construction of ΔNsi I (deletion of the region containing the consensus AP1 site [Fig. 3A]), pBluescript II KS(−) −4.8kb mAP1 FHCAT was digested with NsiI and the larger DNA fragment was isolated and self-ligated.

(iv) Mutants with deletions in the E1A-responsive region. pBluescript II KS(−) −4kbAP1 FHCAT, −4kbAP1+α FHCAT, −4kbAP1+β FHCAT, and −4kbFHAP1+γ FHCAT (Fig. 4A) were constructed by ligation of synthetic double-stranded DNAs (Table 1) to ApaI- and BglII-digested pBluescript II KS(−) −4.8kbFHCAT. pBluescript II KS(−) −4kbAP1+δ, -ζ, and -ε FHCAT plasmids were similarly constructed following ligation of ApaI-BglII-digested, PCR-amplified DNA fragments to ApaI- and BglII-digested pBluescript II KS(−) −4.8kbFHCAT. The 3′ PCR primer for −4kbAP1+δ, -ζ, and -ε (Table 1) was located 0.17 kb 3′ of the BglII site of the mouse ferritin H gene. For construction of ΔXho I/Bgl II (Fig. 4A), pBluescript II KS(−) −4.8kbFHCAT was digested with XhoI and BglII, and this was followed by filling-in with the large fragment of DNA polymerase I and blunt end ligation.

(v) Mutation or deletion of the dyad symmetry sequence from the E1A-responsive element. pBluescript II KS(−) −4.8kbΔDyad FHCAT was constructed by ligation of two PCR-amplified DNA fragments to the ApaI-BglII fragment of pBluescript II KS(−) −4.8kbFHCAT. The 0.8-kb fragment encom-

passing the dyad symmetry sequence and the *Apa*I site in the pBluescript II KS(−) vector was amplified by PCR using Vent DNA polymerase and then digested with *Apa*I. The fragment extending from the dyad symmetry sequence to the *Bgl*II site was similarly amplified and then digested with *Bgl*II, and the smaller DNA fragment was isolated. The primers used in these PCRs are listed in Table 1. The deleted sequence in this construct was the 22-bp dyad symmetry sequence and the proximal GGAGCAGAATGC.

pBluescript II KS(−) −4.8kbmDyad FHCAT was constructed by PCR primer-mediated mutagenesis using the primers shown in Table 1 to produce two PCR products of 0.82 and 0.48 kb. Following denaturation, reannealing, and amplification using outside primers, the 1.3-kb product was digested with *Apa*I and *Bgl*II and ligated to the *Apa*I-*Bgl*II fragment of pBluescript II KS(−) −4.8kbFHCAT. This procedure introduced five nucleotide changes into the dyad symmetry sequence as follows (changes are underlined): TTTGGAGCCCAAC<u>CCCT</u>CCA AA was changed to TTTGGAGCCCAAC<u>TCGGAT</u>AAA.

(vi) Insertion of the E1A-responsive element into CAT reporter plasmids. Each synthetic double-stranded DNA shown in Fig. 6A was cloned into the *Sma*I site (0.22 kb 5′ to the transcription initiation site) of pBluescript II ΔStu I/Eco RV FHCAT. The pCAT-promoter/AP1-like plasmid used for transfection of F9 cells was constructed by tandem insertion of the 16-nucleotide AP1-like element shown in Fig. 6A into the pCAT-promoter plasmid (Promega), which had been digested with *Bgl*II and blunt ended by treatment with the large fragment of DNA polymerase I.

c-*jun* and c-*fos* expression plasmids. The pcDEBΔ vector (34), in which expression is under the control of the SRα promoter, was used for expression of mouse c-*jun* (39) and mouse c-*fos* (kindly provided by M. Green).

DNA sequencing. All plasmid constructs were characterized by restriction enzyme digestion, and the nucleotide sequence of each deleted or mutated region was verified by dideoxy DNA sequencing with a United States Biochemical sequencing kit.

Transient DNA transfection and CAT assay. DNA transfection was carried out by the DEAE-dextran method as described previously (46). Briefly, NIH 3T3 cells were plated at 5×10^5 cells per 60-mm-diameter dish 1 day before transfection. A total of 5 μg of pUC18 or p12SE1A DNA (kindly provided by E. Moran) was mixed with 15 μg of the test plasmid DNA (and 5 μg of pUC18SRαLacZ in some experiments) in 2 ml of serum-free Dulbecco modified Eagle medium containing 0.25 M Tris (pH 7.4) and 400 μg of DEAE-dextran per ml. After incubation at room temperature for 15 to 20 min, the DNA solution was added to the cell monolayer and the cells were incubated at 37°C in 5% CO_2 for 3 to 5 h. The cells were then treated with 100 μM chloroquine in growth medium for 1 h, washed twice with phosphate-buffered saline, and further incubated in growth medium at 37°C in 5% CO_2. After 35 to 48 h, cell extracts were prepared and measured for CAT activity as described previously (46). β-Galactosidase activity was measured as described previously (40). Transfection of test plasmids into F9 cells was carried out by the calcium phosphate precipitation method (11).

Gel retardation assay. Nuclear extracts were prepared from pooled transfectants as described previously (17) with minor modifications. Briefly, confluent cells were suspended in a solution containing 40 mM Tris (pH 7.4), 1 mM EDTA, and 150 mM NaCl and pelleted by centrifugation. The cell pellet was homogenized in solution A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and then briefly centrifuged. The nuclear pellet was then resuspended in 0.5 to 1 ml of solution B (20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 400 mM NaCl, 10% sucrose, 20% glycerol, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and incubated at 4°C for 30 min with occasional rocking. After centrifugation at 14,000 rpm (MRX-152; Tomy Seiko Co.) for 30 min at 4°C, the supernatant was dialyzed against solution C (20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). The concentration of protein in nuclear extracts was measured with the Bio-Rad protein assay kit. Synthetic double-stranded oligonucleotides were end labeled with T4 polynucleotide kinase. Binding reactions were performed at room temperature for 15 min by incubation of 20 μg of nuclear extracts with 1.25 ng of end-labeled probe in 20 μl of solution C containing 1 μg of poly(dI-dC) and 10 μg of bovine serum albumin. A 125-ng portion of unlabeled oligonucleotide was added to some reaction mixtures as a competitor at the same time. When appropriate, 2 μg of anti-c-fos antibody (K25; Santa Cruz Biotechnology) or anti-c-jun antibody (D; Santa Cruz Biotechnology) was added to nuclear extracts 1 h prior to incubation with ^{32}P-labeled probe. Protein-DNA complexes were separated by 5% polyacrylamide gel electrophoresis in TBE buffer (89 mM Tris, 89 mM boric acid, 20 mM EDTA [pH 8.0]). The gels were treated with 10% isopropanol–10% acetic acid for 15 min and then dried and subjected to autoradiography.

RESULTS

The E1A-responsive region of the mouse ferritin H gene is located 4.1 kb upstream from the transcription initiation site. We previously cloned the mouse ferritin H gene and sequenced a part of its 5′ flanking region (25). To identify the E1A-responsive element in the mouse ferritin H gene, deletion analysis using chimeric genes containing variable regions of the ferritin H 5′ flanking region fused to the CAT reporter gene was carried out. As our previous results suggested that transcriptional repression of ferritin H by E1A was mediated through a *cis*-acting element(s) contained within 4.8 kb of the 5′ flanking region (46), this 4.8-kb ferritin H DNA fragment was fused to the CAT gene in the pBluescript II KS(−) vector [forming pBluescript II KS(−) −4.8kbFHCAT; see Materials and Methods]. The DNA of the resulting plasmid was digested with *Apa*I and *Cla*I and then treated with exonuclease III and mung bean nuclease to isolate ferritin H-CAT plasmids containing a series of 5′ deletions (Fig. 1A). We have previously demonstrated that mouse NIH 3T3 cells stably transfected with the adenovirus 13S E1A oncogene show reduced expression of the H subunit of ferritin in the absence of any effect on L subunit expression (46). More recently, we have observed that stable expression of 12S E1A, lacking conserved region 3, also represses ferritin H (data not shown). In the experiments reported here, E1A-mediated repression of chimeric ferritin H-CAT genes was tested by transient cotransfection with p12SE1A into NIH 3T3 cells. In each experiment, pUC18 (the backbone plasmid of p12SE1A [33]) was cotransfected with ferritin H-CAT constructs as a control. pUC18 cotransfection did not have any effect on the activity of the mouse ferritin H promoter compared with transfection of the CAT reporter plasmid alone (data not shown). As shown in Fig. 1B and C, CAT activity of pBluescript II KS(−) −4.8kbFHCAT was approximately 70% repressed by cotransfection with 12S E1A. Similarly, expression of −4.6kbFHCAT, −4.5kbFHCAT, and −4.2kbFHCAT was inhibited by 12S E1A. In contrast, −3.8kbFHCAT transfected into NIH 3T3 cells exhibited lower basal CAT activity than −4.8kbFHCAT, and it was not further inhibited by cotransfection with 12S E1A. Constructs containing further 5′ deletions, such as −3.3kbFHCAT and −1.6kbFHCAT, exhibited behavior similar to that of −3.8kbFHCAT, i.e., decreased basal activity and absence of repression by 12S E1A (Fig. 1B). This was not due to differences in transfection efficiency, since equivalent expression of β-galactosidase was observed when a β-galactosidase expression plasmid (pUC18SRαLacZ) was cotransfected with FHCAT constructs and p12SE1A (or pUC18) (data not shown). These results suggest that basal promoter activity of the mouse ferritin H gene may be controlled, at least in part, by a region between 4.2 and 3.8 kb 5′ of the transcription initiation site and that this region is also a target for E1A-dependent transcriptional repression of the mouse ferritin H gene.

To verify these results, another set of CAT constructs with internal deletions in the 5′ ferritin H gene was constructed and similarly tested for response to E1A (Fig. 2A). ΔKpn I (deletion from −3.4 to −0.29 kb) and ΔBgl II/Eco RV (deletion from −4.0 to −0.32 kb) retained the responsive element for repression by 12S E1A (Fig. 2B). However, FHCAT constructs containing deletions from −4.13 to −0.32 kb (ΔStu I/Eco RV), from −4.2 to −0.29 kb (ΔSst I/Kpn I), and from −4.5 to −0.29 kb (ΔXho I/Kpn I) were unable to be repressed by 12S E1A (Fig. 2B). These results indicate that the E1A-responsive region in the mouse ferritin H gene is located between the *Stu*I and *Bgl*II sites (between 4.13 and 4.0 kb from the transcription initiation site), consistent with the results shown in Fig. 1.

DNA sequence of the region in the mouse ferritin H gene containing the E1A-responsive element. The region of the ferritin H gene containing the E1A-responsive element (*Stu*I to *Bgl*II) was sequenced (Fig. 3A). There is one consensus AP1 site (TGAGTCA) that overlaps an NF-E2 site (GCTGAGT
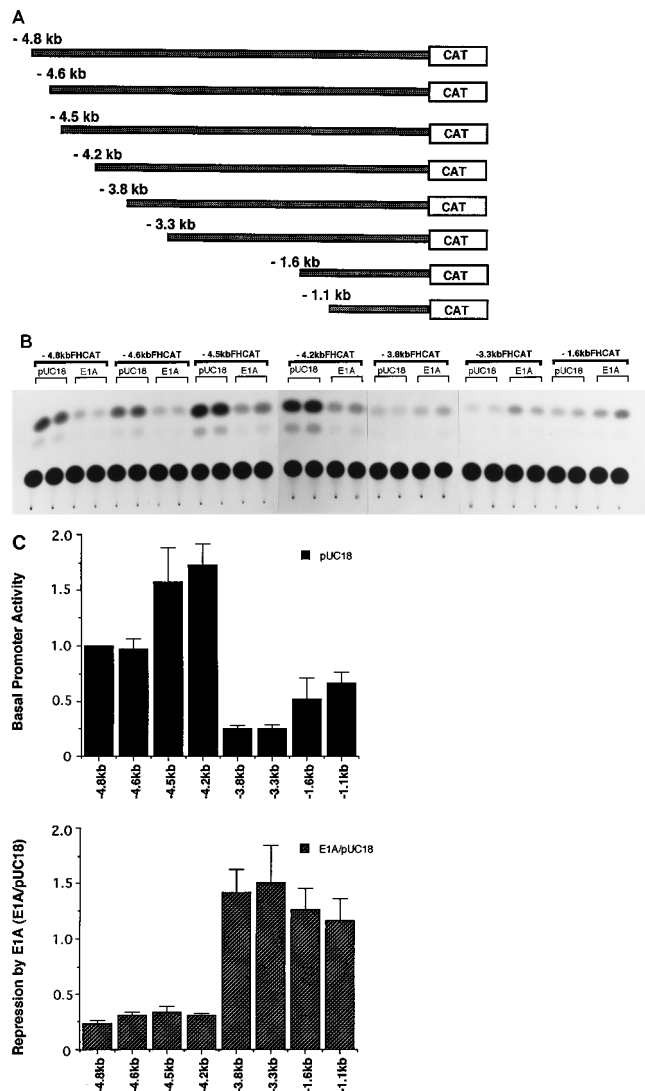
A

B

C

FIG. 1. Mapping of the E1A-responsive element in the 5′ flanking region of the ferritin H promoter by 5′ deletions. (A) Schematic maps of the mouse ferritin H enhancer/promoter-CAT constructs created by exonuclease III-mung bean nuclease digestion. The ferritin H enhancer/promoter is shown as stippled bars. (B) Transient expression of ferritin H enhancer/promoter-CAT constructs following cotransfection with p12SE1A (E1A) or pUC18. NIH 3T3 cells were transfected with 20 μg of each CAT construct and 5 μg of either pUC18 or p12SE1A. Each transfection was carried out in duplicate, and results of a typical CAT assay are shown. (C) Autoradiographs of the thin-layer chromatography plates from three independent experiments (duplicate for each experiment) were quantitated. Percent acetylation of chloramphenicol in extracts from cells cotransfected with −4.8kbFHCAT and pUC18 was defined as 1.0 (upper graph). Repression of ferritin H promoter activity by E1A was obtained by dividing the CAT activity observed following cotransfection of each CAT construct with pUC18 by the CAT activity observed when the same construct was cotransfected with E1A (lower graph). Standard errors are shown.

CAC) in this region. Although this region did not contain any other sequences corresponding to known transcription factor binding sites, we noted that it contained an AP1-like sequence (TGACAAA) followed by a 22-bp dyad symmetry element (TTTGGAGCCCAACCCCTCCAAA) 13 bp upstream from the consensus AP1 site. Another characteristic to be noted is that there is a multisatellite sequence (25 CA repeats) following the *Bgl*II site. However, this repeated sequence is not involved in E1A-mediated repression of the mouse ferritin H gene on the basis of the results shown in Fig. 1 and 2.

The consensus AP1 site in the region containing the E1A-responsive element is not a target of E1A. To test the involvement of the consensus AP1 binding sequence in the repression of the mouse ferritin H gene by E1A, a mutation in this AP1 binding sequence (TGAGTCA→ATCGTCA) was made in pBluescript II KS(−) −4.8kbFHCAT by PCR (producing −4.8kb mAP1 [Fig. 3A]). This mutation caused a substantial decrease (approximately 20-fold) in AP1 binding compared with the original consensus AP1 binding as measured by a gel retardation assay (data not shown). After cotransfection of −4.8kb mAP1 with p12SE1A or pUC18 into NIH 3T3 cells, CAT activity in the cells was measured. As shown in Fig. 3B, the mutation in the AP1 binding sequence caused a slight
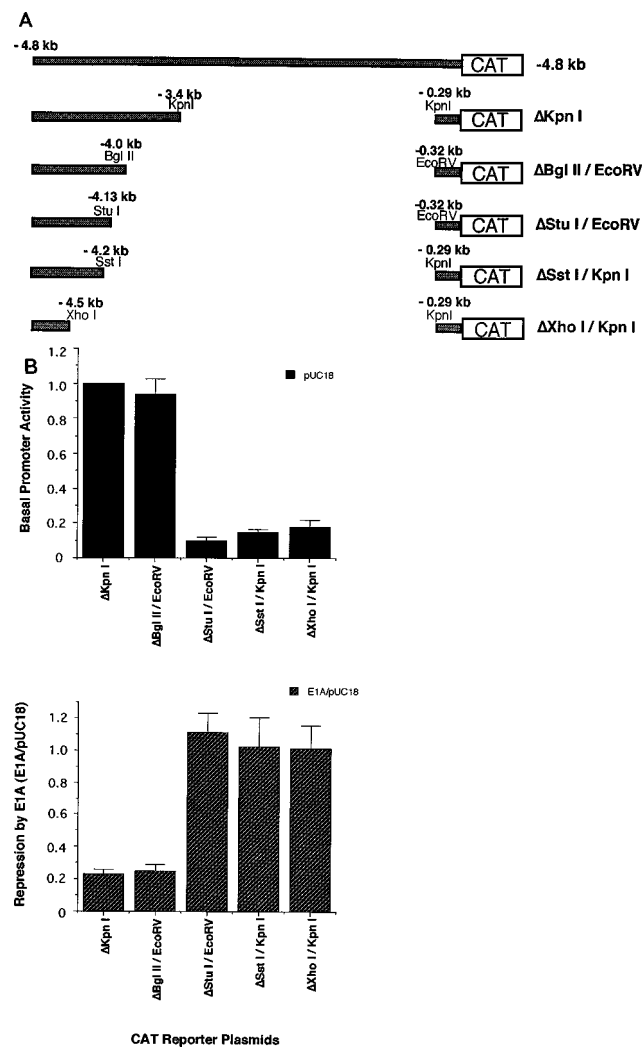
A

B

CAT Reporter Plasmids

FIG. 2. Mapping of the E1A-responsive element in the 5′ flanking region of the ferritin H promoter by internal deletions. (A) Schematic map of internal deletions of the mouse ferritin H enhancer/promoter created with the indicated restriction enzymes. The ferritin H enhancer/promoter is shown as stippled bars. (B) Transient cotransfection of each CAT construct with either pUC18 or p12SE1A was carried out, and autoradiographs of the thin-layer chromatography plates from three independent experiments (duplicate for each experiment) were quantitated. Percent acetylation of chloramphenicol in extracts from cells cotransfected with ΔKpn I and pUC18 was defined as 1.0 (upper graph). Repression of ferritin H promoter activity by E1A was obtained by dividing the CAT activity observed following cotransfection of each CAT construct with pUC18 by the CAT activity observed when the same construct was cotransfected with E1A (lower graph). Standard errors are shown.
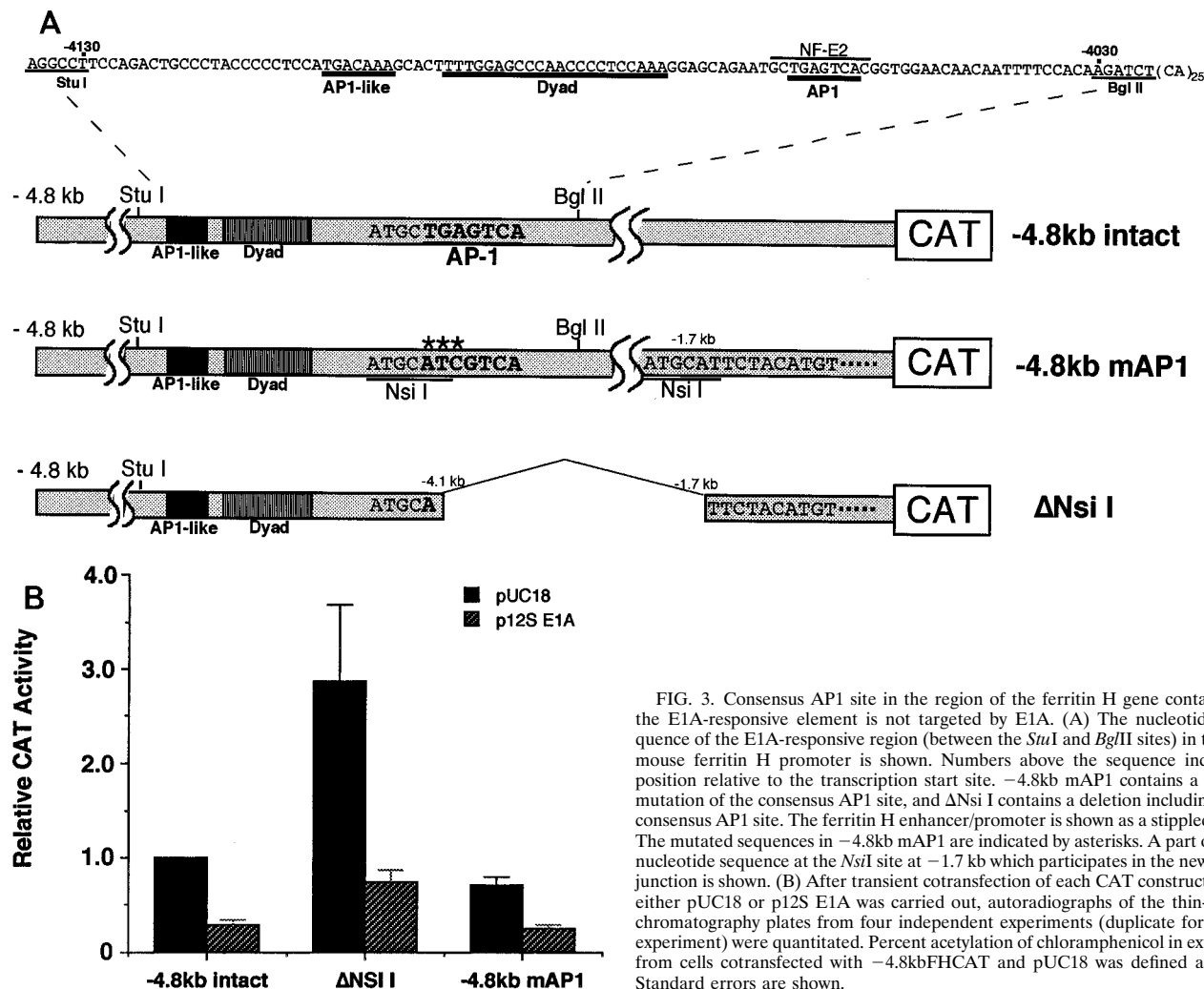
FIG. 3. Consensus AP1 site in the region of the ferritin H gene containing the E1A-responsive element is not targeted by E1A. (A) The nucleotide sequence of the E1A-responsive region (between the *Stu*I and *Bgl*II sites) in the 5′ mouse ferritin H promoter is shown. Numbers above the sequence indicate position relative to the transcription start site. −4.8kb mAP1 contains a point mutation of the consensus AP1 site, and ΔNsi I contains a deletion including the consensus AP1 site. The ferritin H enhancer/promoter is shown as a stippled bar. The mutated sequences in −4.8kb mAP1 are indicated by asterisks. A part of the nucleotide sequence at the *Nsi*I site at −1.7 kb which participates in the new *Nsi*I junction is shown. (B) After transient cotransfection of each CAT construct with either pUC18 or p12S E1A was carried out, autoradiographs of the thin-layer chromatography plates from four independent experiments (duplicate for each experiment) were quantitated. Percent acetylation of chloramphenicol in extracts from cells cotransfected with −4.8kbFHCAT and pUC18 was defined as 1.0. Standard errors are shown.

decrease in basal promoter activity but did not affect E1A-mediated repression of the ferritin H promoter. In addition, by taking advantage of the new *Nsi*I site in the mutated AP1 sequence, a broad region (−4.1 to −1.7 kb) including this consensus AP1 site was deleted (ΔNsi I) and tested in a similar transient transfection assay. As shown in Fig. 3B, promoter activity was still strongly repressed by E1A in this construct. We reproducibly observed an increase in basal CAT activity in ΔNsi I as well as in ΔKpn I and ΔBgl II/EcoRV as shown in Fig. 2. Although we do not have an explanation for this increase (expression of a cotransfected β-galactosidase reporter plasmid rules out differences in transfection efficiency), one possibility is the deletion of a negative regulatory element(s) located in the region extending from the *Kpn*I site at −3.4 kb to the *Nsi*I site at −1.7 kb deleted in these CAT reporter plasmids. These results indicate that the consensus AP1 site does not play an important role in the basal activity of the ferritin H gene or in E1A-mediated repression of ferritin H.

E1A responsiveness requires the AP1-like sequence. To identify essential elements in the E1A-responsive region, a series of plasmids with sequential fine deletions in the region between the *Stu*I and *Bgl*II sites were constructed and tested for their response to E1A in a cotransfection assay (Fig. 4A). In accordance with the results shown in Fig. 3, the consensus

AP1 binding sequence alone (−4kbAP1) was not sufficient to restore basal activity of the ferritin H promoter or repression of this activity by E1A (Fig. 4B). −4kbAP1+α, which has a complete consensus AP1 binding sequence plus an additional 12-bp upstream sequence, similarly did not exhibit a restoration of basal CAT activity or repression by E1A. −4kbAP1+β, which has a part of the 22-bp dyad symmetry element in addition to the sequences present in −4kbAP1+α, still failed to show high basal CAT activity or E1A-mediated repression comparable to that obtained with −4.8kb intact FHCAT. Further inclusion of DNA sequences covering the complete 22-bp dyad symmetry element (−4kbAP1+γ and −4kbAP1+ζ) was not sufficient to restore basal promoter activity and E1A responsiveness. In contrast, when the complete AP1-like sequence in addition to the 22-bp dyad symmetry element was present (−4kbAP1+δ and −4kbAP1+ε), the basal promoter activity was increased and was repressed by E1A (Fig. 4B). These results delimit the 5′ boundary of the E1A-responsive region and indicate that a region including the AP1-like sequence takes part in both the enhancer activity of the ferritin H promoter and the repression of this activity by E1A.

A novel 22-bp dyad symmetry sequence participates in the enhancer activity of the ferritin H promoter. In order to identify the 3′ boundary of the E1A-responsive region, a second
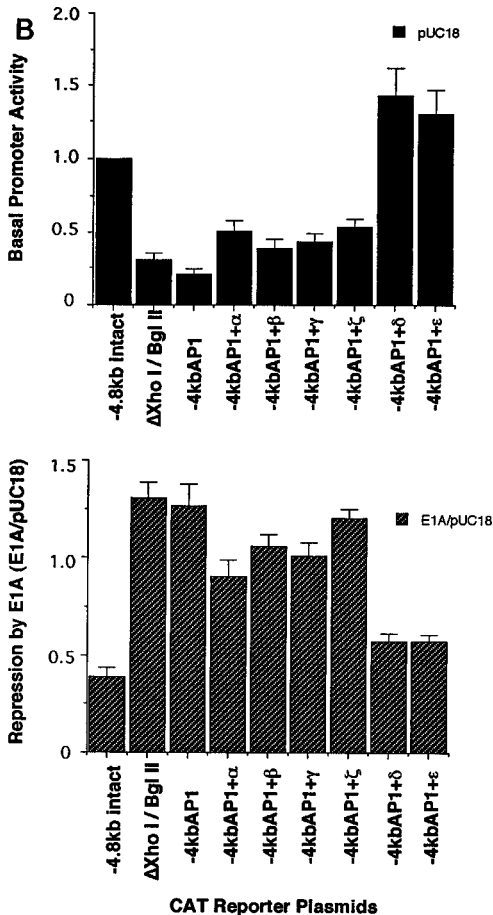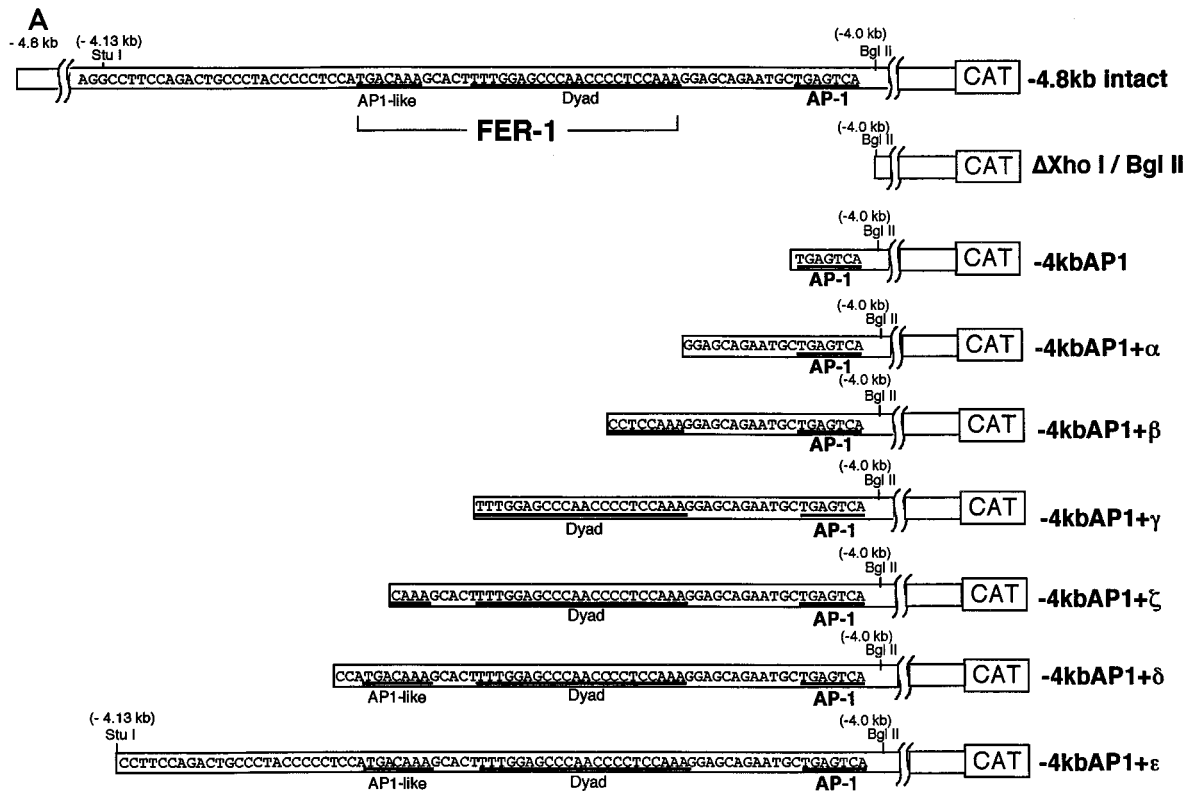
FIG. 4. E1A responsiveness requires the AP1-like sequence. (A) Schematic diagram of ferritin H-CAT constructs used in this experiment. (B) Each CAT construct was transiently cotransfected into NIH 3T3 cells with either pUC18 or p12SE1A. Autoradiographs of the thin-layer chromatography plates from 3 (ΔXho I/Bgl II), 7 (−4kbAP1), 6 (−4kbAP1+α), 5 (−4.8kbAP1+β), 8 (−4kbAP1+γ), 4 (−4kbAP1+ζ), 6 (−4kbAP1+δ and −4kbAP1+), and 11 (−4.8kb intact) independent experiments (duplicate for each experiment) were quantitated. Percent acetylation of chloramphenicol in extracts from cells co-transfected with −4.8kbFHCAT and pUC18 was defined as 1.0 (upper graph). Repression of ferritin H promoter activity by E1A was obtained by dividing the CAT activity observed following cotransfection of each CAT construct with pUC18 by the CAT activity observed when the same construct was cotransfected with E1A (lower graph). Standard errors are shown.
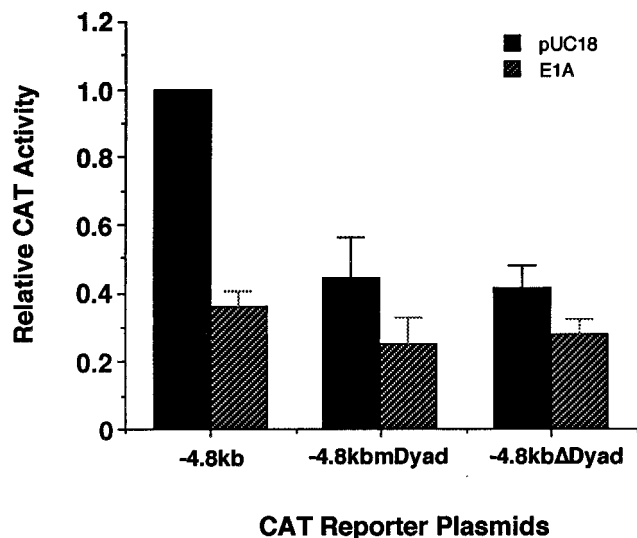
FIG. 5. The 22-bp dyad symmetry element is a component of the E1A-responsive element. The 22-bp dyad symmetry element in −4.8kbFHCAT was mutated (−4.8kbmDyad) or deleted (−4.8kbΔDyad) as described in Materials and Methods and tested for transient expression after cotransfection of pUC18 or p12SE1A. Autoradiographs of the CAT assay thin-layer chromatography plates from six (−4.8kb and −4.8kbΔdyad) and three (−4.8kbmDyad) independent experiments (duplicate for each experiment) were quantitated. Percent acetylation of chloramphenicol in extracts from cells cotransfected with −4.8kbFHCAT and pUC18 was defined as 1.0. Standard errors are shown.

series of plasmids with internal deletions between the StuI and BglII sites of pBluescript II KS(−) −4.8kbFHCAT were constructed and tested for response to E1A. These experiments suggested that full enhancer activity and repression by E1A required the intact dyad symmetry element (data not shown). To confirm the involvement of the 22-bp dyad symmetry element in the ferritin H enhancer activity and E1A-mediated repression, we introduced five nucleotide changes into the dyad symmetry sequence (see Materials and Methods). These changes eliminated the symmetry of the sequence and resulted in a complete loss of its ability to bind nuclear factors (see Fig. 9). As shown in Fig. 5, this mutation (−4.8kbmDyad) diminished the basal activity of the ferritin H promoter by approximately 50% and resulted in markedly reduced responsiveness to E1A-mediated repression. Similar results were obtained with a second mutant, which contained a deletion of the dyad element (−4.8kbΔDyad) (Fig. 5). Thus, the 22-bp dyad symmetry sequence is another key element of the E1A-responsive enhancer of the ferritin H promoter.

The 22-bp dyad element and an AP1-like sequence together compose FER-1, an element responsible for enhancement of ferritin H promoter activity and E1A-mediated repression. To determine whether the AP1-like sequence and the dyad symmetry element were both necessary and sufficient to confer transcriptional enhancement and E1A-mediated repression, three different elements were constructed: 16 bp including the AP1-like sequence alone, the 22-bp dyad symmetry element alone, and 37 bp including both (FER-1). These were inserted into a ferritin H-CAT construct lacking the E1A-responsive region [pBluescript II KS(−)ΔStu I/Eco RV CAT (Fig. 2)] and then cotransfected into NIH 3T3 cells with the p12SE1A or the pUC18 plasmid (Fig. 6A). ΔStu I/Eco RV CAT again showed no repression by E1A (Fig. 6B). When placed as a single copy in the sense orientation into the SmaI site of pBluescript II KS(−)ΔStu I/Eco RV CAT (0.22 kb upstream from the transcription initiation site), the 16-bp DNA containing the AP1-like sequence slightly increased CAT activity driven by the ferritin H promoter, and this activity was repressed by E1A (Fig. 6B, left graph). However, insertion of the 22-bp DNA having only the dyad symmetry element failed to increase ferritin H promoter activity and to confer E1A-mediated repression. In contrast to the case with these elements, insertion of the 37-bp FER-1 element, including both the AP1-like sequence and the 22-bp dyad symmetry element, exhibited a synergistic effect on the activity of the ferritin H promoter and E1A-mediated repression (Fig. 6B, left graph). When two copies of each DNA were inserted into the same site in either the sense-sense orientation (AP1-like sequence or dyad element) or the antisense-antisense orientation (FER-1), similar results were obtained except that a further increase in basal promoter activity was seen, regardless of the orientation of inserted DNA (Fig. 6B, right graph). These results indicate that (i) the 22-bp dyad symmetry element by itself does not have enhancer activity; (ii) a DNA sequence containing an AP1-like site has weak enhancer activity by itself and can be repressed by E1A; (iii) FER-1, composed of the dyad element and the AP1-like element, functions as a classic enhancer, increasing ferritin H transcription independently of position and orientation; and (iv) together, the AP1-like sequence and the dyad symmetry element define a 37-bp region which is necessary for full activity of the ferritin H promoter and repression by E1A.

Interaction of nuclear proteins with components of FER-1. The results of the CAT reporter assays prompted us to investigate the interaction of nuclear proteins with the E1A-responsive element of the mouse ferritin H gene by gel retardation assays. NIH 3T3 cells were stably transfected with E1A or control vector (pHyg), and nuclear extracts were prepared from a pool of over 100 independent clones. Similar results were obtained with extracts from cells transfected with 13S E1A and extracts from cells transfected with 12S E1A. Incubation of these extracts with ^{32}P-end-labeled synthetic DNA of the 22-bp dyad symmetry element produced multiple retarded bands, which were inhibited by an excess amount of unlabeled oligonucleotide corresponding to the 22-bp dyad symmetry element (Fig. 7) but not by oligonucleotides including the proximal consensus AP1 sequence or the AP1-like sequence (data not shown). No difference in specific binding to the 22-bp element was observed in extracts prepared from control or E1A-transfected NIH 3T3 cells (Fig. 7; compare lane a with lane c). When the 16-bp oligonucleotide containing the AP1-like sequence was used as a probe, a slightly diffuse band (possibly representing two bands with very similar migrations), which was inhibited by an excess of the same unlabeled oligonucleotide (lane g) but not by the unrelated NF-κB sequence (data not shown), was reproducibly detected for control vector transfectants (lane e). Nuclear extracts from E1A-transfected cells exhibited reduced binding to this AP1-like sequence (lane f). Taken together, these results suggest that E1A decreases the binding of nuclear factors to the AP1-like sequence of the ferritin H promoter in the absence of any effect on protein binding to the 22-bp dyad symmetry element.

To determine whether members of the AP1 family bind to the AP1-like element of the ferritin H promoter, nuclear extracts from NIH 3T3 cells were preincubated with anti-c-fos or anti-c-jun antibody prior to performance of a gel retardation assay with either the AP1-like element of ferritin H or a consensus AP1 oligonucleotide (Fig. 8). Anti-c-fos and anti-c-jun antibodies used in this experiment were broadly cross-reactive to Fos and Jun family members, respectively. As seen in Fig. 8, when the consensus AP1 sequence was used as a probe, both anti-c-fos and anti-c-jun antibodies altered the mobility of the protein-DNA complex (lanes c and d), whereas control serum
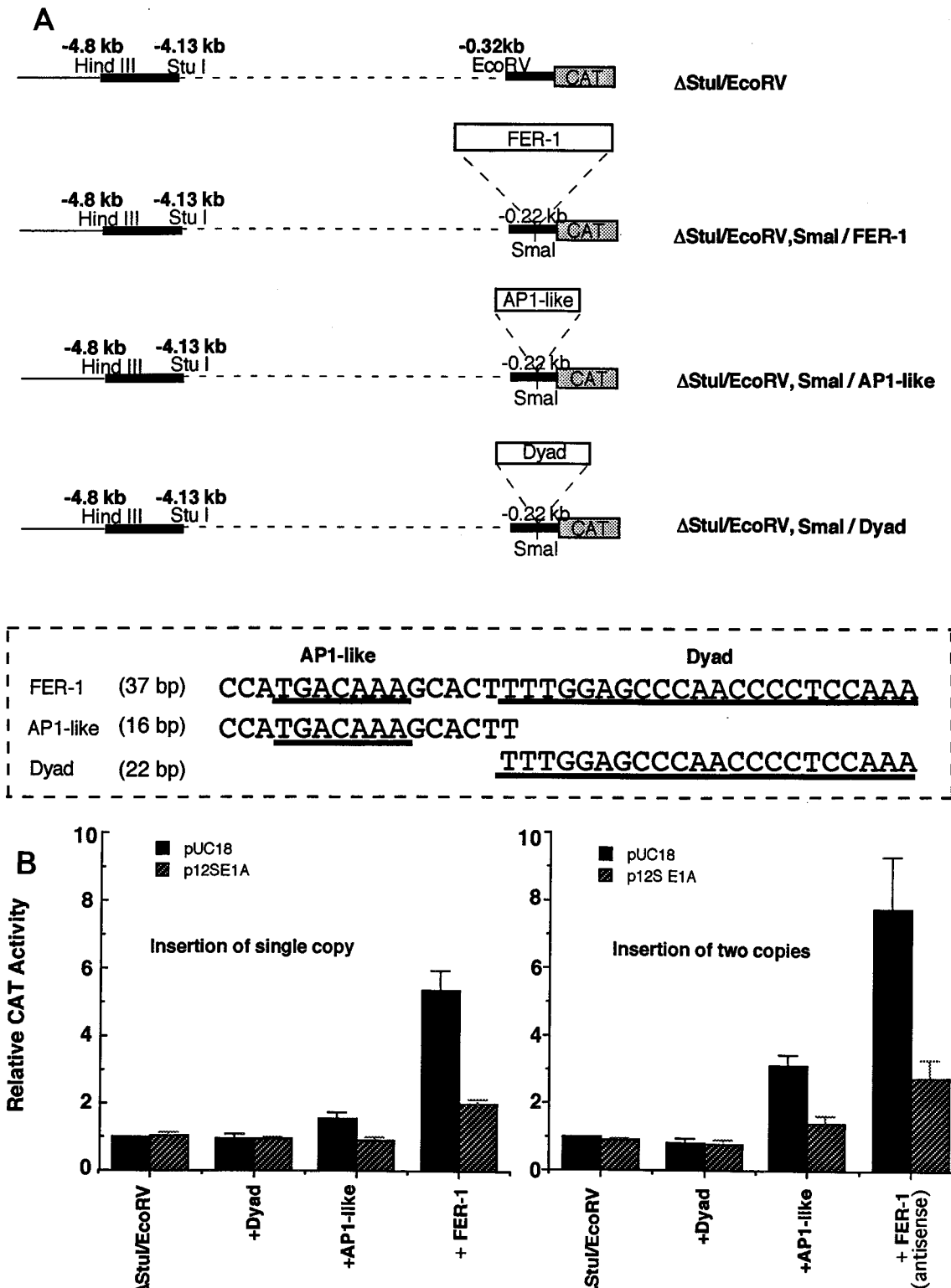
FIG. 6. FER-1 is an enhancer and is responsible for E1A-mediated repression of the ferritin H promoter. (A) FER-1 (comprising the AP1-like sequence and the dyad symmetry element) or its individual component elements were inserted into the *Sma*I site of a ferritin H promoter-CAT construct which lacked the E1A-responsive element (pBluescript II KS(−)ΔStu I/Eco RV) as shown. (B) Each CAT plasmid was cotransfected with either pUC18 or p12SE1A into NIH 3T3 cells. Autoradiographs of the thin-layer chromatography plates from three independent experiments (duplicate for each experiment) were quantitated. The results of the insertion of single copies of individual elements (all in a sense orientation) are shown in the graph on the left, and the results of insertion of two copies of the individual elements (sense-sense orientation in the AP1-like element and the dyad element; antisense-antisense orientation in FER-1) are shown in the graph on the right. Percent acetylation of chloramphenicol in extracts from cells cotransfected with pBluescript II KS(−) ΔStu I/Eco RV CAT and pUC18 was defined as 1.0. Standard errors are shown.
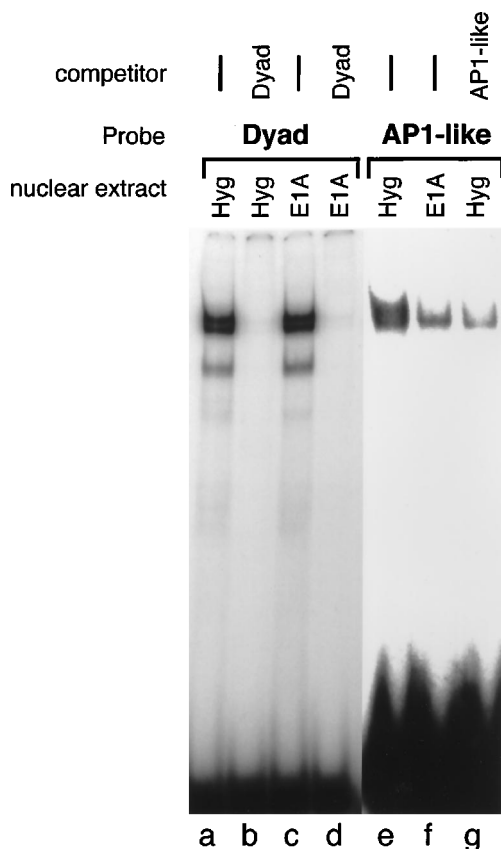
FIG. 7. Characterization of protein(s) binding to components of FER-1 in control and E1A transfectants. Gel retardation assays were carried out with 20 μg of nuclear extracts isolated from pHyg-transfected NIH 3T3 cells (Hyg) or from 13S E1A-transfected NIH 3T3 cells (E1A). Extracts were incubated with a 22-bp dyad symmetry oligonucleotide (Dyad; lanes a to d) or the 16-bp AP1-like oligonucleotide (5'-CCATGACAAAGCACTT-3') (AP1-like; lanes e to g) in the presence of a 100-fold molar excess of an unlabeled oligonucleotide competitor (lanes b, d, and g) or in the absence of competitor.

had no effect (lane b). When the same experiment was carried out with an AP1-like oligonucleotide of identical specific activity, anti-c-fos and anti-c-jun antibodies were again able to alter the mobility of the complexes formed: anti-c-fos antibody blocked the formation of DNA-protein complexes, and anti-c-jun antibody caused a supershift in mobility (lanes g and h). The reduction in band intensity caused by these antibodies was similar to that observed when nuclear extracts from E1A transfectants were used (Fig. 7). These results suggest that members of the Fos and Jun families bind to the AP1-like element of ferritin H.

We next tested the influence of mutations in the dyad symmetry sequence on that sequence's ability to bind nuclear proteins. Destruction of the dyad symmetry by the introduction of five nucleotide changes in the 3′ portion of the 22-bp dyad element was sufficient to abolish the element's ability to bind nuclear factors (Fig. 9). Concomitantly, the ability of the dyad element to contribute to the enhancer activity of the FER-1 element was lost (Fig. 5). Thus, binding of proteins to the dyad symmetry sequence play an important role in the enhancer activity of the ferritin H promoter.

c-fos and c-jun enhance the transcriptional activity of FER-1 in vivo. To determine whether Fos and Jun family members play a role in the regulation of FER-1 in vivo, cotransfection experiments were performed with F9 cells. These

cells contain minimal endogenous AP1 activity compared with other cell types (20). A plasmid containing the AP1-like element of FER-1 ligated to CAT exhibited very low promoter activity when transfected into these cells (Fig. 10). However, the cotransfection of c-*fos* and c-*jun* expression plasmids with this CAT construct greatly enhanced CAT activity (Fig. 10). Thus, the coexpression of c-fos and c-jun enhances the transcriptional activity of the AP1-like element of FER-1.

DISCUSSION

Independent regulation of the H and L subunits of ferritin. Extensive studies of the regulation of ferritin synthesis have been carried out in the past decade. They have revealed that iron, a potent modulator of this major intracellular iron-binding protein, regulates the synthesis of ferritin H and L coordinately at the posttranscriptional level (24). In contrast, it has recently been demonstrated that in response to certain environmental stimuli, ferritin H and L are subject to independent transcriptional control. We have reported that the cytokines tumor necrosis factor alpha and interleukin-1α selectively induce the H subunit of ferritin in several cultured cells (45, 53). These cytokines alter the subunit composition of newly synthesized ferritin molecules (resulting in H-rich ferritins) and may modulate the availability of iron in the cells (30, 47). In addition to these cytokines, thyrotropin, insulin, and cAMP have
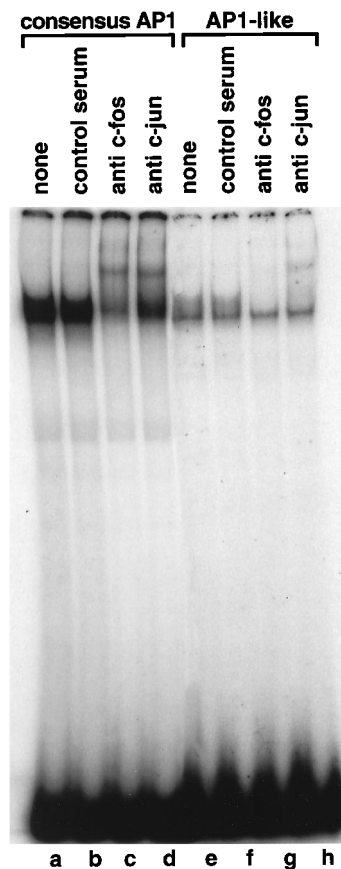

FIG. 8. Effect of anti-c-fos and anti-c-jun antibodies on binding of protein(s) to the AP1-like element. NIH 3T3 nuclear extracts (20 μg) were incubated with the consensus AP1 oligonucleotide (5′-CCATGACTCAGCACTT-3′) (lanes a to d) or the AP1-like oligonucleotide (lanes e to h) in the presence of 2 μg of rabbit whole serum (lanes b and f), anti-c-fos rabbit polyclonal antibody (lanes c and g), or anti-c-jun rabbit polyclonal antibody (lanes d and h).
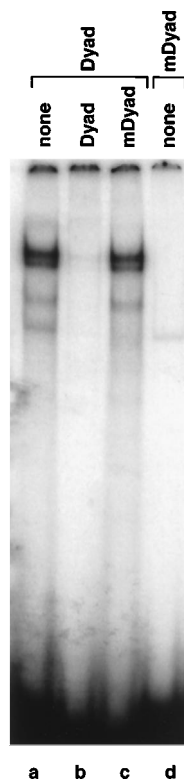
FIG. 9. Mutations in the dyad symmetry sequence abolish its ability to bind nuclear factor(s). NIH 3T3 nuclear extracts (20 μg) were incubated with the 22-bp dyad symmetry oligonucleotide (Dyad; lanes a to c) or the 22-bp mutated dyad symmetry oligonucleotide (mDyad; lane d) alone or in the presence of a 100-fold molar excess of an unlabeled Dyad (lane b) or mDyad (lane c) oligonucleotide. DNA sequences of these oligonucleotides are described in Materials and Methods.

FIG. 10. Expression of c-fos and c-jun augments transcriptional activity of FER-1. Undifferentiated F9 cells were transfected with 15 μg of the pCAT-promoter/AP1-like plasmid and either 5 μg of pcDEBΔ vector (control) or 2.5 μg each of pcDEBΔ c-jun and pcDEBΔ c-fos (c-jun + c-fos). DNA transfection was carried out in duplicate, and results of a typical CAT assay are shown.

been reported by other laboratories to stimulate the transcription of the ferritin H gene in certain cells (13, 27, 56). Such alterations in the subunit composition of the ferritin molecule have the potential to alter intracellular iron balance because of functional differences between the H and L subunits of ferritin: H-subunit-rich ferritin molecules confer a greater ability for rapid iron uptake and release than L-subunit-rich ferritin molecules, which appear to be involved in long-term iron storage (7, 8, 26, 51). Thus, modulation of the H subunit rather than the L subunit of ferritin by cytokines and hormones may represent a cellular response to the requirement for adaptation to acute environmental changes.

FER-1 is the E1A-responsive element of the ferritin H gene. Although alterations in ferritin composition in some tumor cells have been reported (10, 50), the mechanisms underlying this change remain unknown. Using cells transfected with adenovirus E1A, a defined oncogene, we previously observed that E1A preferentially represses transcription of the H subunit of ferritin in mouse NIH 3T3 cells, resulting in ferritin molecules with altered H/L subunit ratios (46). To understand the mechanism by which E1A selectively represses expression of ferritin H, we have studied *cis*-acting elements in the mouse ferritin H gene. The results presented here demonstrate that a 37-bp element, FER-1, which is located approximately 4.1 kb 5′ to the transcription initiation site of the mouse ferritin H gene, is required for E1A responsiveness. FER-1 is composed of two elements, an AP1-like sequence followed by a novel 22-bp dyad symmetry element (Fig. 6A).
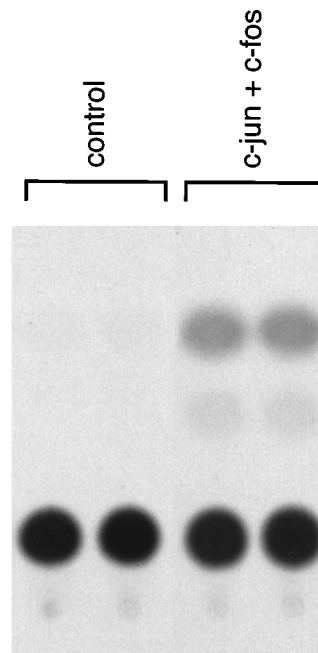
FER-1 differs from sequences in other genes that are targeted for transcriptional repression by E1A. For example, the E1A-responsive element of human collagenase type IV has been mapped to an AP2 site located 1.65 kb upstream from the transcription initiation site (16), whereas repression of human collagenase I by E1A is mediated by E1A-dependent inhibition of binding to an AP1 site (located between 72 and 65 bp upstream from the initiation site) (18). Muscle-specific genes such as those for α-actin, myosin heavy chain, and myogenin are repressed by E1A through a region containing a CArG box (52) or an E box (9), although the binding of proteins to these elements is not affected by E1A. The E1A-responsive element of the H-2Kb promoter of the rat class I major histocompatibility complex gene has recently been mapped to two repeated CAA sequences located 1,725 and 1,568 bp 5′ to the transcription initiation site (37). The rat fibronectin gene is also targeted by E1A for transcriptional repression; in this case, a G10 stretch and two GC boxes present in a 239-bp region of the promoter are the primary E1A-responsive elements (35). E1A transcriptionally represses the rat *neu* oncogene through a TGGAATG sequence localized within a 139-bp DNA fragment in the upstream region of the promoter (57). Similar sequences, $(G)TGG^{AAA}_{TTT}(G)$, can be found in some other genes which can be transcriptionally repressed by E1A, such as immunoglobulin heavy chain and insulin genes (57). Although we detected such a sequence in the promoter of the mouse ferritin H gene, it was not involved in the E1A responsiveness of this gene (data not shown). Including the E1A-responsive element in the ferritin H gene described in this study, it is clear that there is no consensus sequence for E1A-responsive elements. This may imply that E1A-mediated transcriptional repression is achieved by diverse mechanisms which may depend on the target gene.

FER-1 is also an enhancer of the ferritin H gene. In addition to identifying FER-1 as an E1A-responsive element, our stud-

ies have identified it as an enhancer of the mouse ferritin H gene which markedly affects the transcription of this gene in a position- and orientation-independent manner. This enhancer consists of two elements, an AP1-like sequence and a 22-bp dyad symmetry element. The contribution of each of these elements to enhancer activity and E1A response was assessed separately. Our results demonstrate that the 22-bp dyad symmetry element by itself did not show enhancer activity or repression by E1A (Fig. 6). On the other hand, the AP1-like sequence alone had a weak enhancer activity which was repressed by E1A (Fig. 6). However, when both sequences (FER-1) were inserted in either orientation, a potent enhancer activity and strong repression by E1A were observed (Fig. 6). These results suggest that cooperation between the AP1-like motif and the dyad symmetry element is required for maximal enhancer activity.

Although a sequence with some similarity to AP1 is one of the two motifs which compose FER-1, an additional consensus AP1 sequence (TGAGTCA) was found 13 bp downstream from the 22-bp dyad symmetry element in the mouse ferritin H gene. This consensus AP1 sequence does not appear to contribute substantially to E1A-dependent repression of ferritin H, since repression was retained in constructs in which this AP1 site was deleted or mutated (Fig. 3). However, this site may make a minor contribution to ferritin H transcription and repression by E1A, because point mutations in this consensus AP1 binding sequence showed slightly decreased basal CAT activity (−4.8kb mAP1 in Fig. 3). We also noted that an increase in basal promoter activity and repression by E1A was reproducibly observed when a large region 3′ to FER-1 was deleted (ΔKpn I and ΔBgl II/EcoRV in Fig. 2 and ΔNsi I in Fig. 3). Although we do not know the reason for the increase in basal activity caused by these deletions, the deleted regions may contain negative regulatory sequences (possibly similar to the negative regulatory element reported to be present between nucleotides −272 and −291 of the human ferritin H promoter [3]) or the deletions may cause conformational changes which increase the interaction between FER-1 and the promoter region.

E1A-responsive element-binding proteins. To understand the mechanism of E1A-dependent repression of the ferritin H gene, we studied the binding of cellular proteins to FER-1 by gel retardation assays. The results indicated that the binding of cellular proteins to the AP1-like component of FER-1 is reduced by E1A. Binding of protein(s) to the dyad symmetry element was not affected by E1A (Fig. 7). Experiments directed at identifying specific transcription factors that bind to the AP1-like element of FER-1 revealed that the mobility of the complex formed between the AP1-like element and an associated protein factor(s) could be altered with anti-fos and anti-jun antibodies (Fig. 8), suggesting that members of the AP1 family can associate with this element. Further, experiments in which c-*jun* and c-*fos* were cotransfected into F9 cells together with the AP1-like element of FER-1 suggest that increased expression of members of the Fos and Jun families can enhance expression driven by the AP1-like element of FER-1 (Fig. 10). The effect of c-jun and/or c-fos on ferritin expression in these cotransfection experiments may be indirect (i.e., mediated by transcription factors induced by c-fos and/or c-jun rather than by c-fos and/or c-jun directly). Taken together, however, our data support the hypothesis that members of the AP1 family can bind to the AP1-like element of FER-1 and contribute to the role of FER-1 as a transcriptional enhancer of ferritin H and that E1A can interfere with such an interaction. A similar inhibition of interactions between upstream regulatory sequences and AP1 family members in the presence of E1A has been described for the collagenase gene, for which in vivo footprinting demonstrated that E1A selectively inhibits the DNA binding activity of Jun-Jun and Jun-Fos dimeric complexes (18).

It is important to note that our results do not exclude the binding of other proteins (in addition to members of the AP1 family) to FER-1. For example, we observed that in addition to an unlabeled oligonucleotide containing a consensus AP1 site (TGACTCA), an unlabeled oligonucleotide containing TGACGTCA, a consensus cAMP-responsive element, was able to compete with a labeled AP1-like element for binding of nuclear factors (data not shown). Although this result may merely reflect the close similarity between these two sequences (they differ by the presence of one additional nucleotide in the cAMP-responsive element consensus sequence), it may also imply the participation of CREB/ATF family members in ferritin H transcription. Recent observations that CREB and c-jun can heterodimerize (5), and that an E1A-associated protein, p300, has structural and functional similarity to a CREB-binding protein (1, 29), suggest that this possibility deserves further consideration. Clearly, more extensive experiments will be required to fully define the roles of AP1 and CREB/ATF family members in ferritin H transcription.

Role of a novel 22-bp dyad symmetry sequence in FER-1. What is the function of the 22-bp dyad symmetry element in the ferritin H gene? This element by itself does not have enhancer activity, but it augments the enhancer activity of the AP1-like sequence (Fig. 6). In this regard, it resembles other elements which have been reported to function in concert with proximate protein binding sequences. For example, Diamond and colleagues have identified a 25-bp composite glucocorticoid response element in the mouse proliferin gene which includes the glucocorticoid receptor binding site and an AP1 binding sequence (14). Interestingly, glucocorticoids alone have no transcriptional effect through this DNA element in the absence of AP1, suggesting a cooperative function by these two sequences (31). The NF-AT (nuclear factor of activated T cells) binding site of the interleukin-2 gene also contains two essential motifs, AP1 and NF-AT (36, 42). The AP1 sequence functions to facilitate the binding of the NF-AT transcription factor to specific NF-AT DNA sequences through protein complex formation on the NF-AT binding site (6, 21). In the case of FER-1, the binding proteins which recognize the dyad symmetry element and the AP1-like sequence may not interact directly, since preliminary gel retardation assays have shown that the binding of proteins to the intact FER-1 sequence (containing both the AP1-like sequence and the 22-bp dyad symmetry element) could be independently inhibited by an oligonucleotide containing the AP1-like sequence or the 22-bp dyad symmetry element (data not shown). Taken together with the unusually large distance between FER-1 and the ferritin H promoter, this leads us to speculate that a protein(s) which binds to the 22-bp dyad symmetry element may enhance the function of the distal AP1-like binding site through a conformational alteration in ferritin H DNA, possibly rendering this site more interactive with the promoter binding complex or other transcription factors.

This study identifies FER-1 as an enhancer element involved in the transcriptional regulation of the ferritin H gene. FER-1 also serves as a target of E1A-mediated repression. In contrast to E1A-responsive regions described for other genes, FER-1 is located unusually far upstream of the transcription initiation site. Although nuclear proteins bind to both the dyad symmetry element and the AP1-like sequence which compose FER-1, the nature of these proteins remains unknown. Further characterization of the proteins which bind to FER-1 will help elucidate

mechanisms of E1A-mediated transcriptional repression, as well as clarify mechanisms of transcriptional regulation of ferritin expression.

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