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The First Intron of the 4F2 Heavy-Chain Gene Contains a Transcriptional Enhancer Element That Binds Multiple Nuclear Proteins

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We utilized the human 4F2 heavy-chain (4F2HC) gene as a model system to study the regulation of inducible gene expression during normal human T-cell activation. Previous studies have demonstrated that 4F2HC gene expression is induced during normal T-cell activation and that the activity of the gene is regulated, at least in part, by the interaction of a constitutively active 5′-flanking housekeeping promoter and a phorbol ester-responsive transcriptional attenuator element located in the exon 1-intron 1 region of the gene. We now report that 4F2HC intron 1 contains a transcriptional enhancer element which is active on a number of heterologous promoters in a variety of murine and human cells. This enhancer element has been mapped to a 187-base-pair *RsaI-AluI* fragment from 4F2HC intron 1. DNase I footprinting and gel mobility shift analyses demonstrated that this fragment contains two nuclear protein-binding sites (NF-4FA and NF-4FB) which flank a consensus binding site for the inducible AP-1 transcription factor. Deletion analysis showed that the NF-4FA, NF-4FB, and AP-1 sequences are each necessary for full enhancer activity. Murine 4F2HC intron 1 displayed enhancer activity similar to that of its human counterpart. Comparison of the sequences of human and murine 4F2HC intron 1s demonstrated that the NF-4FA, NF-4FB, and AP-1 sequence motifs have been highly conserved during mammalian evolution.

Antigen recognition by the heterodimeric α-β receptor of human T lymphocytes (TCR α-β) results in a complex and precisely orchestrated set of changes in T-cell gene expression, including induction of a set of nuclear proto-oncogenes (19, 33), production of a variety of soluble lymphokines (34), and expression of a novel group of cell surface antigens (9). While a great deal has recently been learned about the structural requirements for antigen recognition by the TCR α-β molecule (for a review, see reference 11), less is understood about the molecular mechanisms involved in modulating inducible gene expression during T-cell activation. Cross-linking of the TCR α-β-CD3 complex has been shown to result in phosphoinositol turnover, protein kinase C activation, and transient increases in cytosolic ionized calcium levels (for a review, see reference 1). How these and other second messengers subsequently regulate T-cell gene expression remains unclear.

We have utilized the 4F2 cell-surface antigen as a model system of gene regulation during human T-cell activation. 4F2 is a 120-kilodalton disulfide-linked heterodimer composed of an 85-kilodalton glycosylated heavy chain (4F2HC) and a 35-kilodalton nonglycosylated light chain (4F2LC) (18). 4F2 is of interest in studies of T-cell activation because it is a T-cell activation antigen which is expressed at low levels on resting peripheral blood T cells and is rapidly induced after activation with lectins or antigen (9). Increases in cell surface expression of 4F2 first occur 4 h after lectin stimulation and peak at 16 to 20 h, before the onset of DNA synthesis or the appearance of the 55-kilodalton interleukin-2 receptor α chain (9). In addition, monoclonal antibodies to 4F2HC are able to block the proliferation of cultures of activated T cells in vitro (10), suggesting that 4F2 is intimately involved in T-cell activation.

The 1.9-kilobase (kb) 4F2HC cDNA encodes a 529-amino-acid type II membrane glycoprotein (32, 39). 4F2HC is encoded by a single-copy human (and murine) gene and does not display significant homology with other known proteins (28a, 32, 39). Our previous studies demonstrated that 4F2HC gene expression is induced 60-fold during normal T-cell activation and that the gene can be activated by both the phorbol ester-inducible protein kinase C pathway and the ionomycin-inducible calcium-mediated activation pathway (16, 25). Studies of the structure of the 4F2HC gene and the molecular mechanisms responsible for 4F2HC gene regulation in resting and activated T cells demonstrated that the gene contains a G+C-rich 5′-flanking housekeeping promoter which lacks TATA and CCAAT sequences but contains binding sites for the ubiquitous Sp1 transcription factor (16). This promoter is equally active in resting and phorbol ester-activated human peripheral blood T cells (25). The low steady-state levels of mature 4F2HC mRNA found in resting T cells were shown to be the result of a block to transcription elongation within the exon 1-intron 1 region of the gene. Phorbol ester activation removes this block to transcription elongation, resulting in increased levels of full-length 4F2HC mRNA (25).

To map the sequences responsible for transcriptional attenuation in the 4F2HC gene, 4F2HC 5′-flanking, exon 1, and intron 1 sequences were introduced into plasmid pSV2CAT (15) containing the bacterial chloramphenicol acetyltransferase (CAT) reporter gene under the control of the simian virus 40 (SV40) promoter-enhancer. Surprisingly, 4F2HC intron 1 was found to contain a potent transcriptional enhancer element which is active in a wide variety of human and murine tissue culture cell lines. We found that this

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enhancer element is active on a number of promoters, including the minimal SV40 promoter, the Herpes simplex virus thymidine kinase (HSVTK) promoter, and the 4F2HC promoter. The enhancer was mapped to a 187-bp *Rsa*I-*Alu*I fragment within 4F2HC intron 1. DNaseI footprinting and gel mobility shift studies revealed that this fragment contains two distinct binding sites for Jurkat nuclear proteins NF-4FA and NF-4FB which flank a consensus binding site for the AP-1 transcription factor. Deletion analyses suggested that the NF-4FA-, NF-4FB-, and AP-1-binding sites are each necessary for full enhancer activity in Jurkat cells. Moreover, a comparison of human and murine 4F2HC intron 1 sequences demonstrated that each of these three sequence motifs has been highly conserved during mammalian evolution.

MATERIALS AND METHODS

Cells. Human cell lines Jurkat (T lymphocytic), K562 (erythroblastic), and clone 13 (B lymphoblastoid) were maintained in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Human HeLa fibroblasts were maintained in minimum essential medium alpha supplemented with 10% fetal calf serum and penicillin-streptomycin.

Plasmids and synthetic oligonucleotides. The plasmids pSV2CAT (15), pUTKAT1 (30), p4F2CAT (16) (containing the human 4F2HC promoter cloned into the *Hin*dIII site of pSV0CAT [15]), and pSPCAT (24) have been described previously. The plasmid pSV2-I_1CAT was constructed by cloning the 500-bp *Stu*I-*Sac*I human 4F2HC genomic fragment (16) (containing the entire 4F2HC intron 1 with small amounts of exon 1 and exon 2 sequences) in a 5' to 3' orientation into the *Hin*dIII site between the SV40 promoter-enhancer and the bacterial CAT gene in pSV2CAT. Plasmids pSV2-I_1Bam/CAT and pSV2-RI_1Bam/CAT were constructed by cloning this same 500-bp fragment in both orientations into the *Bam*HI site located 3' of the CAT gene in pSV2CAT. Plasmids pUTKAT1-I_1 and pUTKAT1-RI_1 were constructed by cloning the same 500-bp *Stu*I-*Sac*I 4F2HC genomic fragment in both orientations into the *Bam*HI site located 5' of the HSVTK promoter in pUTKAT1. Plasmid p4F2-I_1Bam/CAT was constructed by cloning the same 4F2HC genomic fragment into the *Bam*HI site located 3' of the CAT gene in p4F2CAT. Plasmid pSPI_1CAT was constructed by cloning the same 4F2HC genomic fragment into the *Sma*I site located 5' of the minimal SV40 promoter in pSPCAT (24) (see Fig. 1 for schematic representations of these plasmids). The 159-bp *Xho*II-*Nar*I, 129-bp *Hae*II, 213-bp *Kpn*I-*Nar*I, and 187-bp *Rsa*I-*Alu*I fragments from human 4F2HC intron 1 were cloned into the *Bam*HI site of pSV2CAT to create the pSV2-Xho/Nar cat, pSV2-Hae cat, pSV2-H3/Nar cat, and pSV2-Alu/Rsa cat plasmids (see Fig. 4).

Plasmids pSV2 NF4FA$^-$cat, pSV2 NF4FB$^-$cat, and pSV2 AP1$^-$cat were created by deleting the NF-4FA-, NF-4FB-, and AP-1-binding sites from the pSV2-Alu/Rsa cat plasmid with gapped heteroduplex, oligonucleotide-mediated, site-directed mutagenesis (20) involving the following oligonucleotides: NF-4FA$^-$, CATTGTCCTTCCCTCAAGCCGACC CGCCCC; NF-4FB$^-$, GCGGCGCCAGAAGCCATGAAGT AATGTGCAGGACT; and AP-1$^-$, CGTCACGAGGGTGG GGTCCTCCTTCCCCGC. The sequence of each of the mutants was confirmed by the dideoxy method (35) directly from the double-stranded plasmids with a synthetic oligonucleotide primer located 3' of the *Bam*HI site in pSV2CAT (GTTTGTCCAAACTCATC). All oligonucleotides were synthesized on an Applied Biosystems model 380B DNA synthesizer.

CAT assays. All cell lines were transfected, by the DEAE-dextran method as previously described (16), with 10 μg of the appropriate plasmids purified by banding in CsCl. In some experiments, transfection frequencies were monitored by cotransfection with 2 μg of plasmid pRSVGH, a derivative of the previously described plasmid p0GH (37), which contains the Rous sarcoma virus long terminal repeat driving transcription of the human growth hormone gene. Cell extracts were prepared 48 h after transfection by four cycles of freeze-thawing, and protein content was determined by a commercially available assay (Bio-Rad Laboratories, Richmond, Calif.). CAT assays were performed on cell extracts normalized for protein content by using standard techniques and ascending thin-layer chromatography (TLC) as previously described (16). All experiments were repeated at least three times to ensure reproducibility of both the transfections and the CAT assays. In some experiments, in order to equalize for differences in transfection efficiencies, growth hormone levels were determined from the same extracts used for CAT assays with a commercially available kit (Nichols Institute Diagnostics, San Juan Capistrano, Calif.). After autoradiography, spots were cut from the TLC plates and counted in a liquid scintillation counter.

DNase I footprinting. Nuclear extracts were prepared from exponentially growing Jurkat cells as described by Dignam et al. (12). Fragments from 4F2HC intron 1 were end labeled with [α-^{32}P]dATP (Amersham Corp., Arlington Heights, Ill.) by using the Klenow fragment of DNA polymerase I and were purified on 4% polyacrylamide gels. Labeled DNA fragment (30,000 cpm) was incubated in the presence of 63 μg of Jurkat nuclear extract and 1 μg of PolydI:dC (Pharmacia, Piscataway, N.J.) for 20 min at 22°C essentially as described by Lee et al. (22). Reaction mixtures were digested with 0.2 to 0.9× DNase I (1× DNaseI is a final concentration of 17 U/ml) (Worthington Diagnostics, Freehold, N.J.) for 30 s at 22°C. Digestions were stopped by adding 1 volume of 8 M urea–0.5% sodium dodecyl sulfate–5 mM EDTA followed by phenol extraction, and the DNase I digestion products were resolved by electrophoresis in 8% polyacrylamide–6 M urea sequencing gels. Standard Maxam-Gilbert purine-sequencing reaction mixtures (26) were prepared and electrophoresed adjacent to the footprinting reactions in each experiment.

Gel mobility shift assays. Gel mobility shift assays were performed as described by Singh et al. (38). Complimentary oligonucleotides corresponding to both strands of the NF-4FA (CCCTCCTTTCTTTGAAGAAAGCCGA)-, NF-4FB (GCGCCAGAAGCCAGTTGCAACCGGTTTCTGAAG)-, and AP-1 (AGGGTGGGTGACTCAGCGTCA)-binding sites from human 4F2HC intron 1 were synthesized with overhanging *Bam*HI and *Bgl*II ends, annealed, end labeled with [α-^{32}P]dATP by using the Klenow fragment of DNA polymerase I, and purified by electrophoresis on 10% polyacrylamide gels. Binding reaction mixtures contained 20,000 cpm of gel-purified oligonucleotide (0.1 to 0.5 ng), 0.5 μg of polydI:dC, and 4.7 μg of Jurkat nuclear extract in 4% glycerol–1 mM EDTA–5 mM dithiothreitol–10 mM Tris (pH 7.5)–100 mM NaCl–100 μg of bovine serum albumin per ml. Binding reactions were carried out at room temperature for 15 min, and the fragments were resolved on 4% polyacrylamide gels (acrylamide:N,N^1-methylenebisacrylamide ratio, 30:1) in Tris-glycine buffer (50 mM Tris, 0.38 M glycine,

0.002 M EDTA [pH 8.5]) run at 100 V for 2 to 3 h. Gels were dried and subjected to autoradiography with intensifying screens (Du Pont Co., Wilmington, Del.). For cold competition experiments, binding reactions took place in the presence of 10 to 50 ng of cold competitor double-stranded oligonucleotide.

Cloning and sequencing of murine 4F2HC intron 1. A murine 4F2HC genomic clone was identified by hybridization of 10^6 recombinant clones from a murine genomic library (Clonetec, Palo Alto, Calif.) to a radiolabeled murine 4F2HC cDNA (28a). A 1.35-kilobase *Bam*HI restriction enzyme fragment from this genomic 4F2HC clone which contained intron 1 was identified by hybridization to a synthetic oligonucleotide probe (ATCGGCGACCTTCA GGC) homologous to the 3′ end of the 4F2HC exon 1 sequence (the position of which was determined from the human 4F2HC genomic structure [16]) and subcloned into pUC18. Murine 4F2HC intron 1 was sequenced directly from double-stranded DNA by using the dideoxy-chain termination method (35) and synthetic oligonucleotide primers corresponding to the 3′ end of murine 4F2HC exon 1 (ATCGGC GACCTTCAGGC) and the 5′ end of murine 4F2HC exon 2 (GCCCTTCACCTTCAGGG) as well as to a sequence from the middle of the intron (GGGTGCCTGACTCAGCA and TGCTGAGTCAGGCACCC) determined by using the exon primers as described above. The entire intron (including the intron-exon boundaries) was sequenced on both strands. The human and murine intron 1 sequences were compared by using the ALIGN program of the DNASTAR software package (DNASTAR, Madison, Wis.).

RESULTS

4F2HC intron 1 contains a transcriptional enhancer element. In an attempt to map the sequences responsible for the previously reported (25) transcriptional attenuator activity of the 4F2HC gene exon 1-intron 1 region, a genomic fragment containing 4F2HC intron 1 was subcloned into the *Hin*dIII site of plasmid pSV2CAT (15) between the SV40 promoter-enhancer and the bacterial CAT reporter gene (Fig. 1A). This construct, along with the control plasmid pSV2CAT, was transfected into human Jurkat T cells by using DEAE-dextran (16), and transcriptional activity was monitored by CAT assay (Fig. 1A). The SV40 promoter-enhancer was relatively inactive in Jurkat cells, as evidenced by the low CAT activity produced by transfection of the control plasmid pSV2CAT. Surprisingly, plasmid pSV2-I_1CAT, containing the 500-bp 4F2HC genomic fragment which includes the entire 4F2HC intron 1 surrounded by short regions of exon 1 and exon 2 sequences to allow for accurate splicing (Fig. 1A, pSV2-I_1CAT), displayed CAT activity 60-fold higher than that of plasmid pSV2CAT alone (Fig. 1A). To determine whether this effect was due to an enhancer element or to a cryptic promoter within this region of the 4F2HC gene, the same fragment was subcloned in both orientations into the *Bam*HI site which lies 3′ of the bacterial CAT gene in pSV2CAT (Fig. 1A, pSV2-I_1Bam/CAT and pSV2-RI_1Bam/CAT). Both constructs expressed 40 to 45 times more CAT activity than did control plasmid pSV2CAT (Fig. 1A), indicating that this fragment contains a potent transcriptional enhancer element. To determine whether the 4F2HC enhancer required the presence of the SV40 enhancer element or was active on the minimal SV40 promoter, the 500-bp 4F2HC intron 1 fragment was cloned 5′ of the minimal SV40 promoter in plasmid pSPCAT (24) and transfected into Jurkat cells (Fig. 1A). pSPI_1CAT containing the 4F2HC intron 1 fragment expressed 35 times more CAT activity than did the control plasmid pSPCAT, indicating that the 4F2HC enhancer was active on the minimal SV40 promoter in the Jurkat cells. In a parallel series of experiments, 4F2HC intron 1 was also shown to enhance transcription from both the 4F2HC and HSVTK promoters, resulting in 5- to 10-fold increases in the activity of these promoters in Jurkat cells (Fig. 1B and C).

To determine the cellular specificity of this enhancer, pSPCAT and pSPI_1CAT were used to transfect a variety of human cell lines, including K562 erythroleukemia cells, clone 13 B cells, and HeLa fibroblasts. In all cases, 4F2HC intron 1 was able to increase transcription from the minimal SV40 promoter by 5- to 30-fold (Fig. 2). In addition, human 4F2HC intron 1 was also able to significantly increase transcription from the minimal SV40 promoter in murine EL4 T cells and L-cell fibroblasts (data not shown). Together, these results indicated that intron 1 of the 4F2HC gene contains a transcriptional enhancer element which can function with a number of heterologous promoters in an orientation- and position-independent fashion in a wide variety of cell types. The potency of this enhancer in T cells is remarkable; it is able to significantly increase transcription from the SV40 promoter even in the presence of the SV40 enhancer element (Fig. 1A).

Mapping the 4F2HC enhancer. To more precisely map the 4F2HC enhancer element, a variety of restriction enzyme fragments from 4F2HC intron 1 were subcloned into the *Bam*HI site 3′ of the bacterial CAT gene in pSV2CAT and assayed for their ability to enhance CAT transcription in Jurkat cells (Fig. 3). Fragments from the 5′ half of the intron (bp 81 to 240; pSV2-Xho/Nar cat) or the 3′ half of the intron (bp 243 to 372; pSV2-Hae cat) produced by digestion with *Xho*II-*Nar*I or HaeII displayed a marked reduction in enhancer function, although both fragments retained the ability to enhance CAT expression by 10- to 15-fold compared with the control plasmid pSV2-cat. In contrast, the 187-bp *Alu*I-*Rsa*I fragment from the middle of the intron (bp 110 to 297; pSV2-Alu/Rsa cat) displayed full enhancer activity (Fig. 3). These results demonstrated that while all the enhancer sequences map to a relatively small fragment from the center of the intron, sequences on both sides of the *Nar*I site (bp 240) are needed for full enhancer activity. Thus, either the *Nar*I site lies in the middle of a single enhancer motif or there are multiple enhancer motifs which flank this site.

The 4F2HC enhancer contains two novel nuclear protein-binding sites which flank a consensus binding site for the AP-1 transcription factor. A number of viral and cellular transcriptional enhancer elements have been shown to contain specific binding sites for nuclear transcriptional regulatory proteins (for reviews, see references 14, 27, and 31). To identify putative 4F2HC enhancer nuclear protein-binding sites, restriction enzyme fragments from 4F2HC intron 1 were subjected to DNaseI footprint analyses by using nuclear extracts prepared from Jurkat T cells (Fig. 4). Two sequence motifs (NF-4FA and NF-4FB) within the *Alu*I-*Rsa*I enhancer fragment were specifically protected by Jurkat nuclear proteins. These sites were protected on both strands. In addition to the NF-4FA and NF-4FB sites, an 18-bp sequence (NF-4FC) directly 5′ of the AP-1 site was partially protected by Jurkat nuclear proteins (Fig. 4).

Interestingly, the NF-4FA- and NF-4FB-binding sites surround a consensus binding site for the AP-1 transcription factor (3, 22). While the AP-1 site was not protected by unstimulated Jurkat nuclear extracts, the DNase I cleavage pattern of the site was subtly altered in the presence of

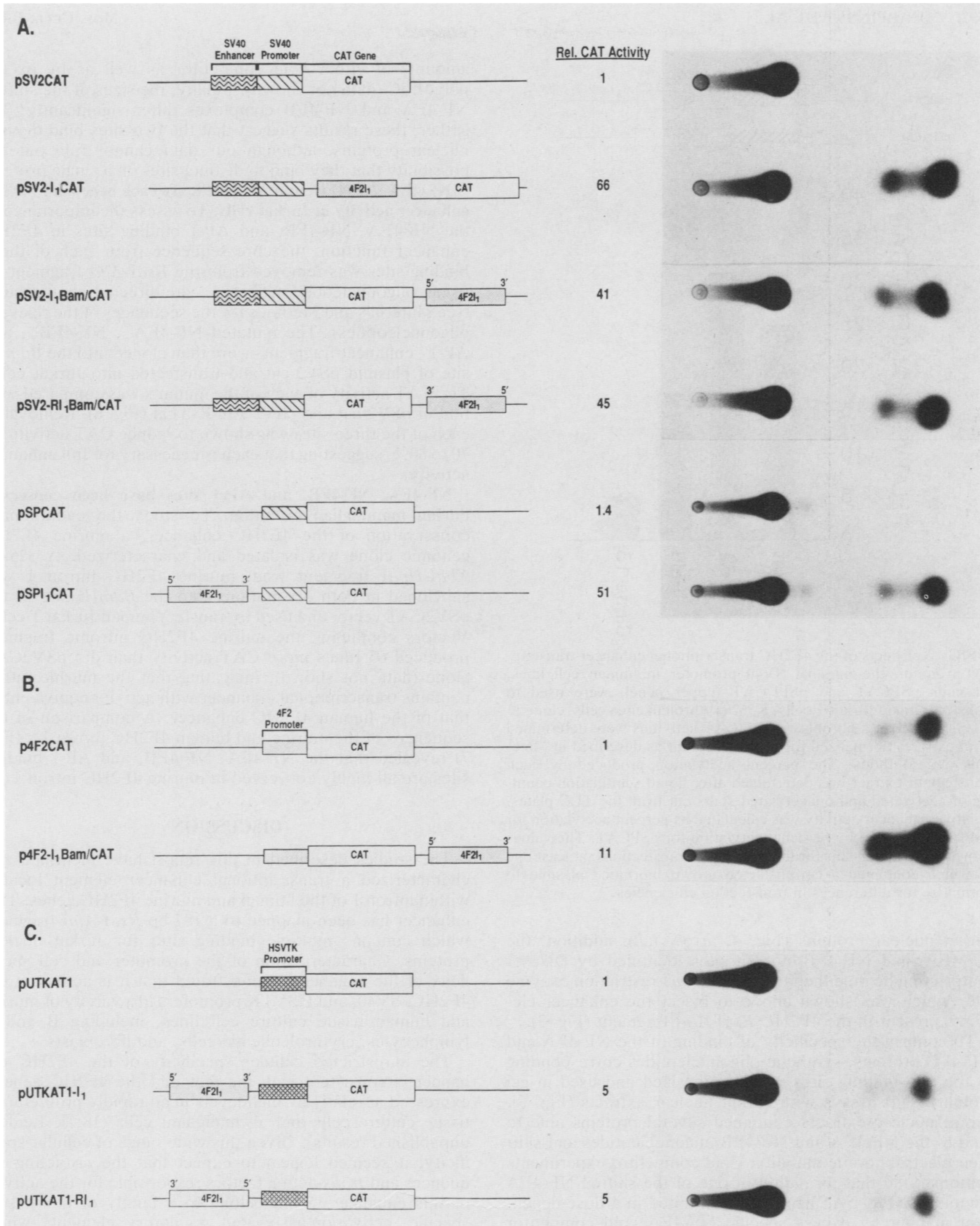

FIG. 1. Effects of 4F2HC intron 1 on the transcriptional activity of three different promoters. The 500-bp *Stu*I-*Sac*I fragment containing human 4F2HC intron 1 ($4F2I_1$) (16) was cloned in one or both orientations into the appropriate restriction endonuclease sites either 5′ or 3′ of the bacterial CAT gene in the pSV2CAT, pSPCAT, p4F2CAT, or pUTKAT1 vectors as described in Materials and Methods. The SV40 promoter, SV40 enhancer, 4F2HC promoter (4F2 promoter), and HSVTK promoter are labeled. CAT reporter plasmids containing 4F2HC intron 1 in combination with the SV40 promoter-enhancer or minimal SV40 promoter (A), the 4F2HC promoter (B), or the HSVTK promoter (C) were transfected into Jurkat cells by using DEAE-dextran. CAT activity was determined 48 h after transfection by standard techniques involving ascending TLC. Following autoradiography, spots were cut from the TLC plates and quantitated in a liquid scintillation counter. Relative amounts of acetylation (normalized to the control plasmids pSV2CAT, pSPCAT, p4F2CAT, or pUTKAT1) are shown as relative CAT activity.

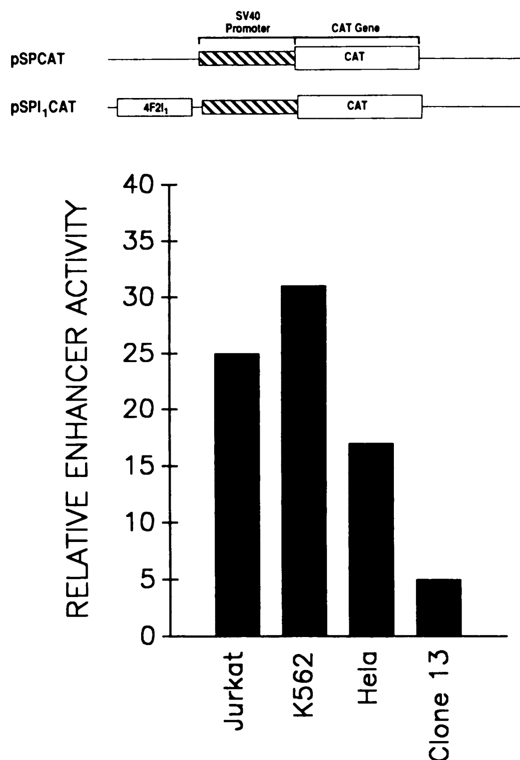

FIG. 2. Effects of the 4F2HC transcriptional enhancer transcription from on the minimal SV40 promoter in human cell lines. Plasmids pSPCAT and pSPI$_1$CAT (upper panel) were used to transfect human Jurkat T cells, K562 erythroleukemia cells, clone 13 B cells, and HeLa fibroblasts, and CAT activities were determined from extracts normalized for protein content as described in Materials and Methods. The percent acetylation produced by each transfectant extract was determined after liquid scintillation counting of acetylated and unacetylated spots cut from the TLC plates. Relative enhancer activity was calculated as percent acetylation for pSPI$_1$CAT divided by percent acetylation for pSPCAT. The values shown represent means for three separate transfections, at least one of which contained a cotransfected growth hormone plasmid to normalize for differences in transfection efficiencies.

Jurkat nuclear proteins (Fig. 4, arrows). In addition, the NF-4FA- and NF-4FB-binding sites identified by DNaseI footprinting lie on either side of the *Nar*I restriction enzyme site, which was shown above to bisect the enhancer elements present in the 4F2HC *Rsa*I-*Alu*I fragment (Fig. 3).

To confirm the specificity of binding of the NF-4FA and NF-4FB proteins, synthetic oligonucleotides corresponding to the two binding sites were synthesized and used in gel mobility shift assays with Jurkat nuclear extracts (Fig. 5). Jurkat nuclear extracts contained several proteins able to bind to the NF-4FA and NF-4FB oligonucleotides and shift their electrophoretic mobility. Cold competitor experiments demonstrated that formation of one of the shifted NF-4FA complexes (Fig. 5A, arrow) was inhibited in a dose-dependent manner by excess specific NF-4FA cold competitor oligonucleotide but not by an identical amount of cold NF-4FB competitor. Similarly, formation of one of the shifted NF-4FB complexes (Fig. 5B, arrow) was inhibited by excess cold NF-4FB competitor but not by an identical amount of cold NF-4FA competitor. The shifted complexes of lower molecular weights which were present in both binding experiments appear to represent nonspecific binding, as their formation was partially inhibited by excess amounts of either cold competitor as well as by excess polydI:dC (data not shown). Finally, the sizes of the shifted NF-4FA and NF-4FB complexes differ significantly. Together, these results suggest that the two sites bind distinct nuclear proteins, although our data cannot rule out the possibility that they bind to distinct sites on a single protein.

NF-4FA, NF-4FB, and AP-1 sites are each necessary for full enhancer activity in Jurkat cells. To assess the importance of the NF-4FA, NF-4FB, and AP-1 binding sites in 4F2HC enhancer function, the core sequence from each of these binding sites was removed from the *Rsa*I-*Alu*I fragment by using oligonucleotide-mediated, site-directed mutagenesis (see Materials and Methods for the sequences of the relevant oligonucleotides). The mutated NF-4FA$^-$, NF-4FB$^-$, and AP-1$^-$ enhancer fragments were then cloned into the *Bam*HI site of plasmid pSV2-cat and transfected into Jurkat cells. The CAT activity of each of the mutants was compared with that of pSV2-cat and pSV2-Alu/Rsa cat (Fig. 6). Removal of each of the three sites was shown to reduce CAT activity by 70 to 80%, suggesting that each is necessary for full enhancer activity.

NF-4FA, NF-4FB, and AP-1 sites have been conserved during mammalian evolution. To study the evolutionary conservation of the 4F2HC enhancer, a murine 4F2HC genomic clone was isolated and characterized. A 325-bp *Xba*I-*Hpa*I fragment from murine 4F2HC intron 1 was subcloned in both orientations into the *Bam*HI site of the pSV2CAT vector and used to transfect human Jurkat T cells. Vectors containing the murine 4F2HC intronic fragment produced 65 times more CAT activity than did pSV2CAT alone (data not shown), indicating that the murine intron contains transcriptional enhancer with activity equivalent to that of the human 4F2HC enhancer. A comparison of the sequences of the murine and human 4F2HC intron 1's (Fig. 7) revealed that the NF-4FA, NF-4FB, and AP-1 binding sites are all highly conserved in murine 4F2HC intron 1.

DISCUSSION

The studies described in this report have identified and characterized a transcriptional enhancer element located within intron 1 of the human and murine 4F2HC genes. This enhancer has been mapped to a 187-bp *Rsa*I-*Alu*I fragment which contains multiple binding sites for Jurkat nuclear proteins. Characterization of the promoter and cell specificity of the enhancer demonstrated that it is active on the 4F2HC, SV40, and HSVTK promoters in a variety of murine and human tissue culture cell lines, including B and T lymphocytes, erythroleukemia cells, and fibroblasts.

The unrestricted cellular specificity of the 4F2HC enhancer is consistent with the fact that the 4F2HC gene is expressed at relatively high levels in all rapidly proliferating tissue culture cells and all malignant cells (18; J. Leiden, unpublished results). Given this wide range of cellular specificity, it seemed logical to expect that the *cis*-acting sequences and *trans*-acting factors responsible for the activity of this enhancer would belong to a family of non-tissue-specific, activation-associated regulatory elements which might also regulate the expression of other activation-associated genes. However, searches of the Genbank sequence data base (version 56) with the 4F2HC enhancer sequence and the NF-4FA- and NF-4FB-binding sites have thus far failed to identify regions of significant similarity within the 5′-flanking regions or introns of other known genes. Because the 4F2HC enhancer is able to significantly increase levels of gene expression from both the SV40 promoter-enhancer and

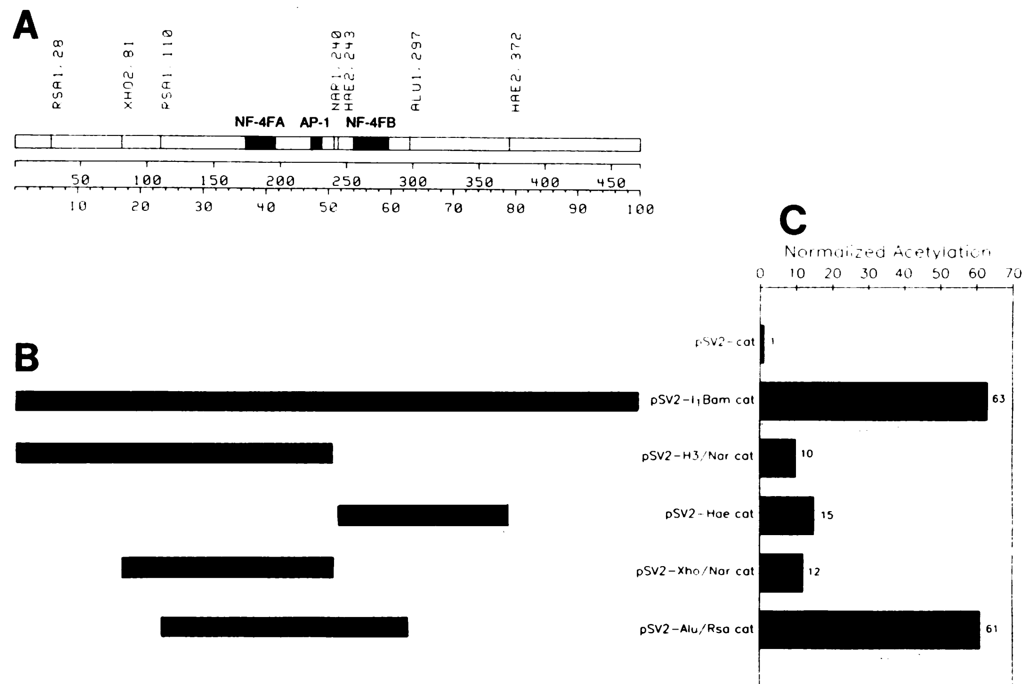

FIG. 3. Mapping of the 4F2HC intron 1 enhancer element. Restriction enzyme fragments derived from human 4F2HC intron 1 were cloned into the *Bam*HI site 3′ of the bacterial CAT gene in plasmid pSV2CAT. These constructs were used to transfect human Jurkat cells, and CAT activity was determined as described in Materials and Methods. The pSV2-cat and pSV2-I_1Bam cat plasmids are identical to the pSV2CAT and pSV2-I_1Bam/CAT plasmids shown in Fig. 1A. (A) Partial restriction endonuclease map of human 4F2HC intron 1. The NF-4FA and NF-4FB nuclear protein DNA-binding sites determined by DNase I footprinting (Fig. 4) are shown schematically, as is the potential binding site for the AP-1 transcription factor (3, 22). The length in base pairs is depicted immediately below the map, and the percent fragment length is shown on the bottom scale. (B) Restriction enzyme fragments (bars) used in the CAT constructs. (C) CAT activity produced by each of the constructs (compared with the control pSV2-cat plasmid, which was normalized to a value of 1), shown as normalized acetylation.

the HSVTK promoter in a variety of tissue culture cell lines, it has proved to be a useful adjunct in several generalized eucaryotic expression vectors, particularly in T-cell transfection vectors which utilize the SV40 promoter-enhancer (B. Karpinski and J. Leiden, unpublished results).

There are at least three distinct *cis*-acting sequences (NF-4FA, NF-4FB, and AP-1) within the 4F2HC enhancer which bind Jurkat nuclear proteins; each appears to be necessary for full enhancer activity. The importance of these sequences in determining enhancer activity is supported both by deletion experiments and by the finding that they have each been highly conserved during mammalian evolution. In addition, we have identified a fourth sequence, NF-4FC, which lies immediately 5′ to the AP-1-binding site and which is less perfectly footprinted by Jurkat nuclear extracts and less highly conserved in mouse 4F2HC intron 1. The importance of the NF-4FC binding site in determining enhancer activity remains unclear. Of note, the locations of these nuclear protein-binding sites correspond to two previously described DNase I-hypersensitive sites located within intron 1 of the human 4F2HC gene in peripheral blood T-cell DNA (16).

The finding of multiple *cis*-acting sequences, each of which is necessary for full 4F2HC enhancer function, is reminiscent of the results of previous studies of the SV40 (8, 13, 28) and immunoglobulin heavy-chain (23, 29) transcriptional enhancers, both of which contain multiple nuclear protein-binding sites which are necessary for full activity. The finding that deletion of the NF-4FA-, NF-4FB-, or AP-1-binding site results in the loss of the majority of enhancer activity suggests that the three binding sites or their cognate nuclear proteins may interact to activate the 4F2HC enhancer. However, it remains possible that deletion of one or more of these sites decreases enhancer activity by altering the geometry of the enhancer rather than by abolishing the binding of its nuclear proteins. These models are being tested by constructing CAT vectors containing various combinations of synthetic oligonucleotides corresponding to each of the 4F2HC enhancer nuclear protein-binding sites.

To our knowledge the NF-4FA and NF-4FB sequences are distinct from previously described enhancer motifs. In contrast, the AP-1/c-*jun* gene product (2, 6) (and its cognate sequence) has previously been shown to be responsible for the phorbol ester inducibility of a number of genes (3, 22), including the human metallothionein II_A gene and the collagenase gene (40). Given our previous finding that the 4F2HC gene is induced by phorbol ester treatment of resting T cells (25) and that this induction is mediated by the removal of a block to transcription elongation within the exon 1-intron 1 region of the gene, it was particularly interesting to identify a consensus binding site for the AP-1 transcription factor within the 4F2HC intron 1 enhancer. Despite our inability to reproducibly footprint the AP-1-binding site with unfractionated Jurkat nuclear extracts, the importance of this site in determining enhancer activity was demonstrated by the finding that its deletion resulted in significant decreases in enhancer activity. However, it remains unclear whether the AP-1 site is also involved in the activation of 4F2HC gene expression during phorbol ester treatment of resting peripheral blood T cells. Although phorbol ester treatment of murine fibroblasts was recently shown to result in induction of the AP-1/c-*jun* gene (21), the effect of phorbol ester

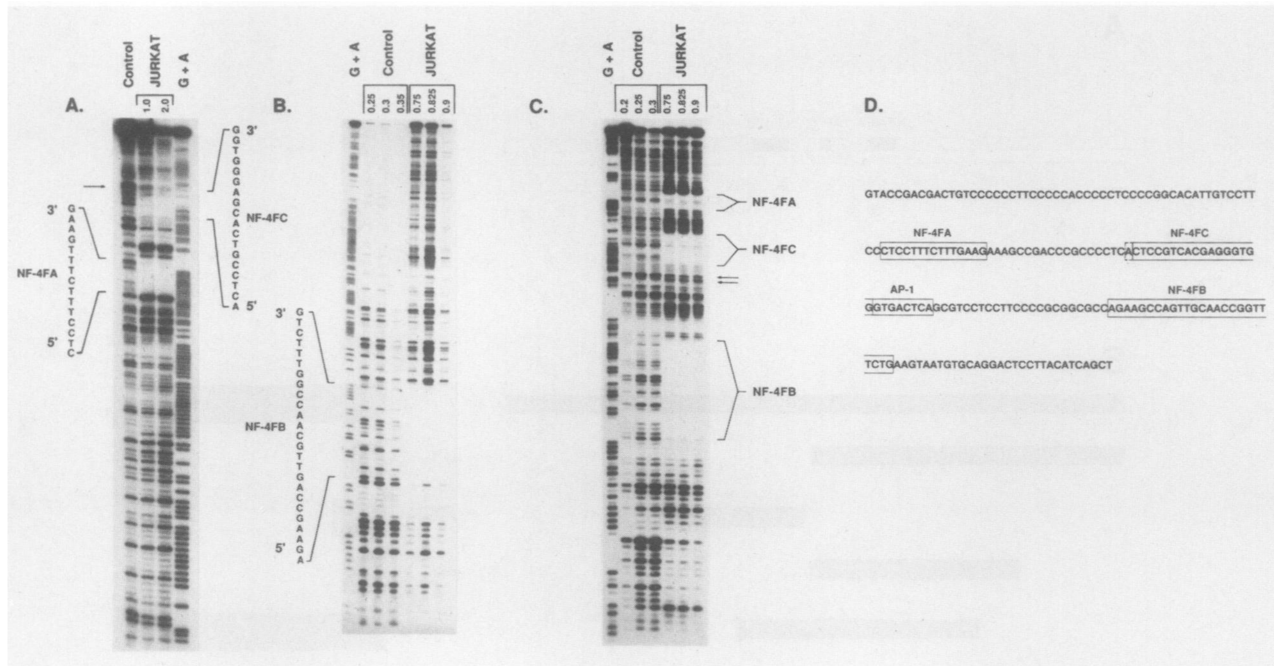

FIG. 4. DNase I footprint analysis of the 4F2HC intron 1 enhancer. ^{32}P-labeled *Kpn*I-*Nar*I (bp 27 to 240) (A), *Nar*I-*Pst*I (bp 240 to 500) (B), or *Rsa*I-*Alu*I (bp 110 to 297) (C) restriction endonuclease fragments from the 500-bp human 4F2HC intron 1 (16) (Fig. 8) were incubated in the presence (Jurkat) or the absence (control) of Jurkat nuclear extract. Protein-DNA complexes were digested for 30 s at room temperature with 0.25 to 2× DNase I (shown at the top of each lane), and the resulting samples were fractionated on 8% polyacrylamide–6 M urea DNA-sequencing gels. Standard Maxam-Gilbert purine reactions (G+A) of the same fragments were run in parallel to identify the footprinted sequences. The NF-4FA and NF-4FB sequences are shown to the left of each panel of gels. Arrows denote subtle changes in band intensities seen in the AP-1-binding site during footprinting with Jurkat nuclear extracts. (D) Sequence of the 187-bp *Rsa*I-*Alu*I fragment from intron 1 of the human 4F2HC gene (16). The NF-4FA-, NF-4FB-, NF-4FC-, and AP-1-binding sites are labeled. The dashed box at the 5′ end of the NF-4FC sequence denotes the variable footprinting of this base.

treatment of resting T cells on AP-1/c-*jun* gene expression is unknown.

Together with our previous studies (16, 25), the results presented in this report demonstrate that 4F2HC gene expression is highly regulated by a unique combination of molecular mechanisms which include (i) a 5′-flanking, constitutively active promoter which contains a calcium-responsive element; (ii) a block to transcription elongation within the exon 1-intron 1 region of the gene which can be modulated by phorbol ester treatment; and (iii) a potent transcriptional enhancer element which is contained in a 187-bp fragment from intron 1 of the gene (Fig. 8). While several other genes have been shown to be regulated by transcriptional attenuation or to have intronic transcriptional enhancer elements, to our knowledge the 4F2HC gene is the first example of a gene which combines these two regulatory elements. It is tempting to speculate on the functional relation of the 4F2HC transcriptional attenuator and enhancer elements. Preliminary analyses indicate that the sequences responsible for transcriptional attenuation of 4F2HC gene transcription may map to the 5′ end of exon 1 (data not shown). It is possible that inactivity of the 4F2HC enhancer in resting T cells results in an increased probability of premature transcription termination, possibly due to an exon 1 sequence element which is difficult for the RNA polymerase II complex to traverse. T-cell activation (via the protein kinase C pathway) might result in enhancer activation, which, in turn, might stabilize the RNA polymerase complex, resulting in transcriptional read-through and increased levels of mature 4F2HC mRNA. Alternatively, it remains possible that attenuator and enhancer activity are

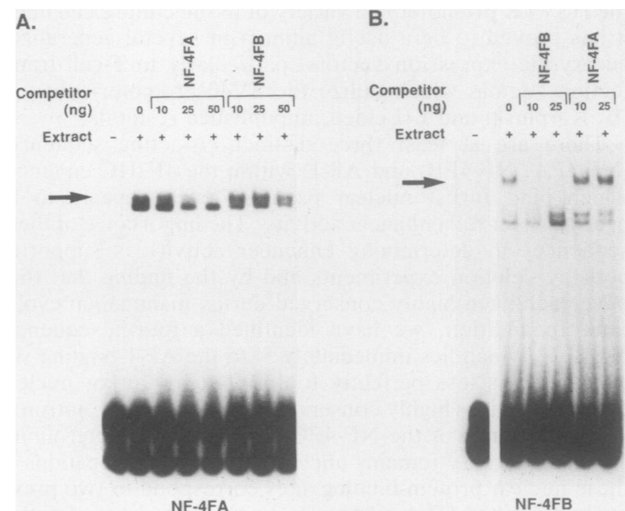

FIG. 5. Gel mobility shift analysis of the NF-4FA and NF-4FB nuclear DNA-binding proteins. ^{32}P-labeled double-stranded synthetic oligonucleotides corresponding to the NF-4FA- and NF-4FB-binding sites as determined by DNase I footprinting (see Fig. 4 and Materials and Methods) were incubated with Jurkat nuclear extracts and fractionated on nondenaturing 4% polyacrylamide gels. In cold competitor experiments, complexes were allowed to form in the presence of 10 to 50 ng of unlabeled double-stranded NF-4FA or NF-4FB competitor oligonucleotide. Arrows indicate the positions of bands of altered mobility which result from specific interactions of Jurkat nuclear proteins and the NF-4FA and NF-4FB oligonucleotides.

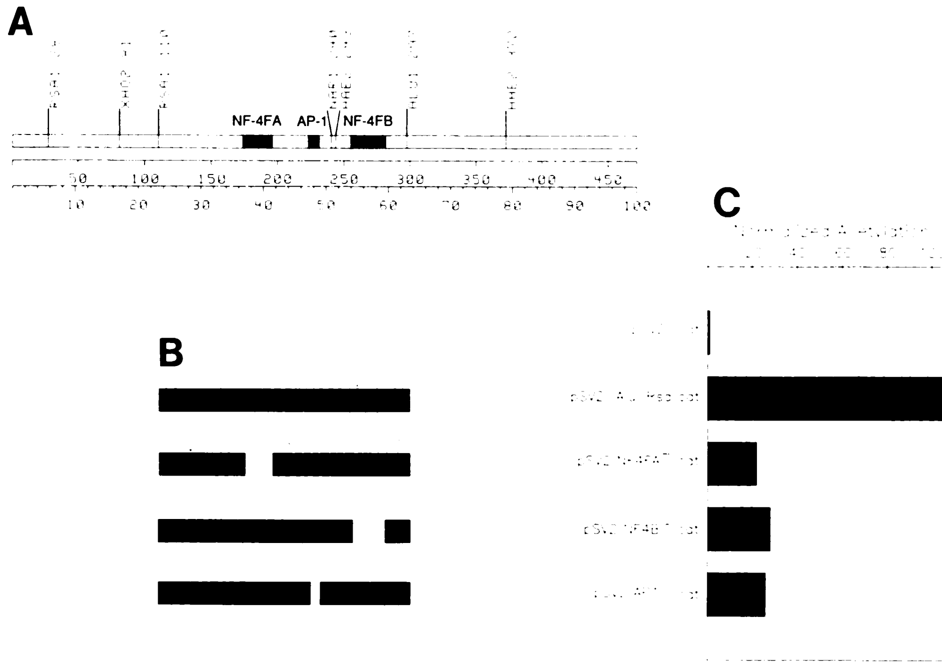

FIG. 6. Effects of NF-4FA-, NF-4FB-, and AP-1-binding site deletions on 4F2HC transcriptional enhancer activity. The NF-4FA-, NF-4FB-, and AP-1-binding sites were deleted from plasmid pSV2-Alu/Rsa cat (Fig. 3) by oligonucleotide-mediated, site-directed mutagenesis to yield pSV2 NF4FA$^-$cat, pSV2 NF4FB$^-$cat, and pSV2 AP1$^-$cat, respectively. These plasmids, along with the control plasmids pSV2-cat and pSV2-Alu/Rsa cat, were transfected into Jurkat cells, and CAT activity was determined as described in Materials and Methods. CAT activities for each transfection were compared with that for the pSV2-cat plasmid transfection, which was normalized to a value of 1. The pSV2-cat plasmid is identical to the pSV2CAT plasmid shown in Fig. 1A. (A) Partial restriction endonuclease map of human 4F2HC intron 1 (bp 1 to 455), with the NF-4FA-, NF-4FB-, and AP-1-binding sites labeled. The length in base pairs is shown immediately below the map, and the percent fragment length is shown on the bottom scale. (B) 4F2HC sequences included in each construct. (C) Normalized CAT activity.

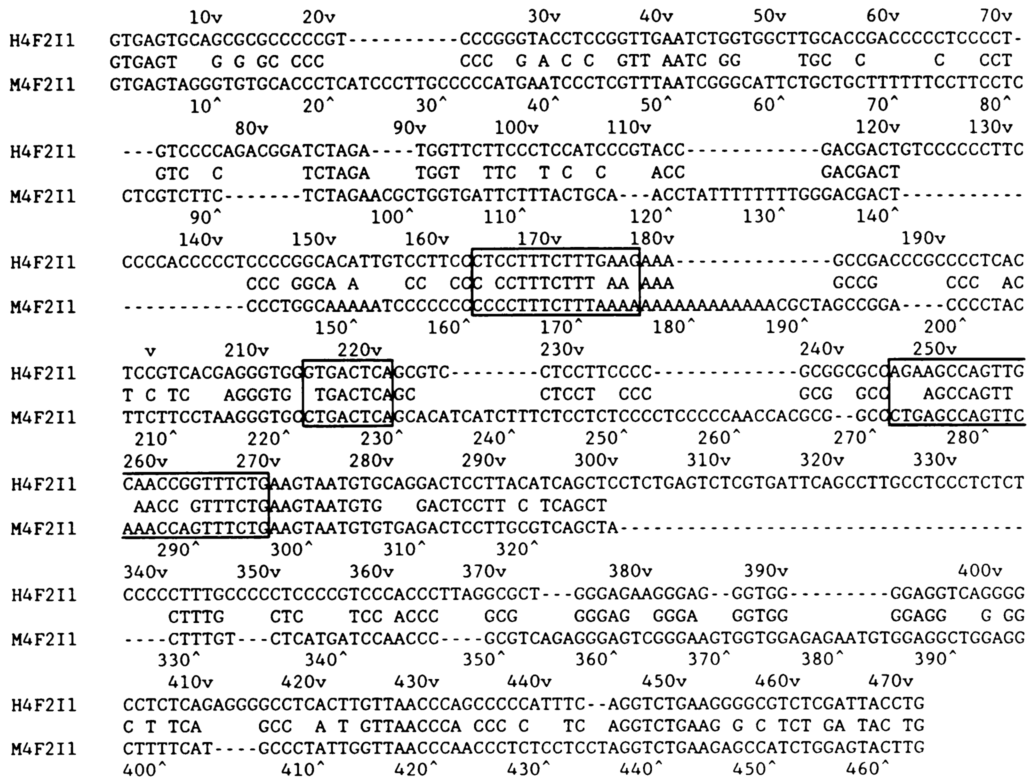

FIG. 7. Sequences of the human and murine 4F2HC intron 1s. Murine 4F2HC intron 1 (M4F2I1) was cloned and sequenced as described in Materials and Methods. Human 4F2HC intron 1 spans bp 1 to 455. Human 4F2HC exon 2 begins at bp 456. The human (H4F2I1) and murine sequences were compared by using the ALIGN program of the DNASTAR sequencing software. Dashed lines indicate nucleotides absent from one of the sequences. Nucleotides shared by both sequences are indicated between them. The NF-4FA (bp 163 to 177)-, NF-4FB (bp 245 to 269)-, and AP-1 (bp 214 to 221)-binding sites are boxed.

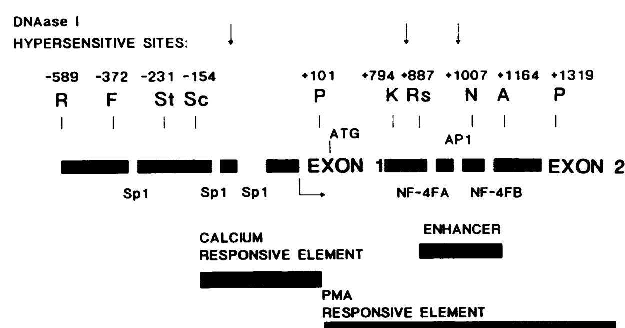

FIG. 8. Schematic illustration of the regulatory elements of the human 4F2HC gene. DNase I-hypersensitive sites within the 4F2HC gene in peripheral blood T-cell DNA are indicated by arrows in the top line (16). Relevant *Eco*RI (R), *Fok*I (F), *Stu*I (St), *Sca*I (Sc), *Pst*I (P), *Kpn*I (K), *Rsa*I (Rs), *Nar*I (N), and *Alu*I (A) restriction endonuclease sites as well as their relative map positions are shown in the second line (16). The exon-intron structure of the gene as well as potential binding sites for the Sp1, AP-1, NF-4FA, and NF-4FB nuclear DNA-binding proteins are shown in the third line. The horizontal arrow indicates the transcription initiation site (16). The locations of the previously reported calcium-responsive element and phorbol myristate acetate (PMA)-responsive element (transcriptional attenuator) (25) and of the newly described transcriptional enhancer element are shown as bars at the bottom.

mediated by the interaction of distinct sets of *trans*-acting factors with a single set of intron 1 *cis*-acting sequences.

In testing these models, it will be of interest to determine whether there are differences in the DNA-binding activities or mRNA levels of the NF-4FA, NF-4FB, and AP-1/c-*jun* gene products in activated versus resting T cells. In addition, because T-cell activation has been shown to increase c-*fos* mRNA levels (17, 33) and because the c-*fos* gene product was recently shown to associate with and activate the AP-1 transcription factor (7, 36), it is possible that de novo c-*fos* transcription and translation are necessary for 4F2HC enhancer activation. This hypothesis is in accord with our previous finding of a requirement for protein synthesis in 4F2HC gene activation in T cells (25). Finally, it will be of interest to determine whether the c-*myc* and c-*myb* proto-oncogenes which have recently been reported to be regulated by transcriptional attenuation (4, 5) also contain intragenic enhancer elements, i.e., whether transcriptional attenuators are commonly associated with or regulated by intragenic transcriptional enhancers. While further studies will be needed to precisely identify the role of the 4F2HC enhancer element in regulating 4F2HC gene expression, the identification and characterization of this enhancer should facilitate future experiments designed to better understand the biochemical pathways involved in regulating inducible gene expression during T-cell activation.

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