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Transcriptional Activation of the *fra-1* Gene by AP-1 Is Mediated by Regulatory Sequences in the First Intron

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Constitutive expression of c-Fos, FosB, Fra-1, or c-Jun in rat fibroblasts leads to up-regulation of the immediate-early gene *fra-1*. Using the posttranslational FosER induction system, we demonstrate that this AP-1-dependent stimulation of *fra-1* expression is rapid, depends on a functional DNA-binding domain of FosER, and is a general phenomenon observed in different cell types. In vitro mutagenesis and functional analysis of the rat *fra-1* gene in stably transfected Rat-1A–FosER fibroblasts indicated that basal and AP-1-regulated expression of the *fra-1* gene depends on regulatory sequences in the first intron which comprise a consensus AP-1 site and two AP-1-like elements. We have also investigated the transactivating and transforming properties of the Fra-1 protein to address the significance of *fra-1* up-regulation. The entire Fra-1 protein fused to the DNA-binding domain of Gal4 is shown to lack any transactivation function, and yet it possesses oncogenic potential, as overexpression of Fra-1 in established rat fibroblasts results in anchorage-independent growth in vitro and tumor development in athymic mice. *fra-1* is therefore not only induced by members of the Fos family, but its gene product may also contribute to cellular transformation by these proteins. Together, these data identify *fra-1* as a unique member of the *fos* gene family which is under positive control by AP-1 activity.

Eukaryotic cells communicate via extracellular signals that interact with specific cell surface receptors and thus initiate a cascade of biochemical events leading to selective regulation of gene expression. A variety of extracellular signals rapidly and transiently stimulates the activity of the transcription factor AP-1, which is thought to play a central role in the reprogramming of gene expression in response to external stimuli. AP-1 consists of a complex mixture of polypeptides that are encoded by the immediate-early genes of the *fos* and *jun* families. The three Jun proteins (c-Jun, JunB, and JunD) and the four Fos family members (c-Fos, FosB, Fra-1, and Fra-2) share a homologous region containing the basic DNA-binding domain and the leucine zipper dimerization motif. Jun proteins are able to form homo- and heterodimers, while Fos proteins are only capable of forming heterodimeric complexes with Jun proteins. Both Jun and Fos contribute to the transactivation function of the AP-1 complex, which stimulates transcription by binding to AP-1 consensus sequences (TGA G/C TCA) in enhancer and promoter regions. AP-1 is also capable of repressing gene activity through negative protein-protein interactions with other transcription factors (reviewed in references 1, 29, and 43).

The transcription factor AP-1 has been implicated in diverse cellular processes including cell proliferation, differentiation, and neuronal function (reviewed in reference 1). In particular, ectopic expression of certain members of the *fos* and *jun* families results in the transformation of fibroblasts, supporting a possible role for these genes in cell proliferation (35, 53, 68). Analysis of c-*fos*-deficient mice has, however, indicated that c-*fos* is not required for the proliferation of most cell types (5, 25, 64). Instead, c-*fos* is essential for differentiation of bone-resorbing osteoclasts, as mice lacking c-*fos* suffer primarily from osteopetrosis (18, 25, 64). Moreover, ectopic expression of c-*fos* in transgenic mice results in bone and cartilage tumors (19, 45, 62) and in altered maturation of T lymphocytes (46). In contrast, no effect of c-Jun overexpression is seen in transgenic mice (19), although c-Jun is essential for normal mouse development and hepatogenesis (22, 26).

Insights into the molecular mechanisms of AP-1 function have been obtained by the identification of genes that are controlled by this transcription factor. To date, more than 20 different AP-1 target genes have been characterized by defining functional AP-1 elements in their control regions by in vitro mutagenesis and transient transfection experiments (reviewed in reference 6). As an alternative approach to the identification of AP-1 target genes, we have developed estrogen-dependent transcriptional and posttranslational Fos induction systems which allow selective activation of AP-1 by providing the limiting Fos component to cycling cells (4, 55). These induction systems were successfully applied to the identification and characterization of several genes which are regulated by AP-1 activity in Rat-1A fibroblasts and PC12 pheochromocytoma cells (2, 3, 6, 69). Moreover, the posttranslational FosER induction system was used to study the effects of inducible c-Fos activity in different cell types, thus demonstrating that FosER leads, in a strictly estrogen-dependent manner, to transformation of fibroblasts (55), to blockade of neuronal differentiation of PC12 cells (69), and to epithelial-mesenchymal conversion of mammary epithelial cells (44).

Using these induction systems, we have previously identified *fra-1* as a Fos-responsive gene in rat fibroblasts (3, 4). *fra-1* was originally isolated as an immediate-early gene which encodes a Fos-related antigen cross-reacting with c-Fos antibodies (9).

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Serum growth factors stimulate transcription of this gene with delayed and protracted kinetics relative to those of c-*fos* and *fosB* induction (9, 31). The *fra-1* gene is also expressed at significant levels in proliferating cells, in contrast to c-*fos* and *fosB*, and injection of neutralizing antibodies against Fra-1 was shown to partially block DNA synthesis in cycling cells, indicating that Fra-1 may be essential for cell cycle progression (31). Fra-1 binds to the different Jun proteins and stabilizes the AP-1 complex in a manner similar to that of other members of the Fos family (10, 48). Recently, Wisdom and Verma (68) demonstrated that Fra-1, unlike c-Fos and FosB, lacks a C-terminal transactivation domain. Moreover, no transforming potential of Fra-1 could be detected in rat fibroblasts by a focus formation assay, in clear contrast to results for c-Fos and FosB (68). In addition to AP-1, the testis-determining factor Sry (11) and the transcription factor Tax-1 of the human T-cell leukemia virus type 1 (58) have recently been shown to stimulate *fra-1* gene expression. However, the molecular mechanism by which these transcription factors regulate the *fra-1* gene is still largely unknown.

Here we demonstrate that *fra-1* is the only member of the AP-1 gene family that is induced by elevated AP-1 activity in rat fibroblasts. Transcriptional stimulation of *fra-1* by estrogen-activated FosER is rapid, occurs in different cell types, and depends on a functional DNA-binding domain of the FosER protein. Regulation of the *fra-1* gene by FosER is mediated by sequences in the first intron which include a consensus AP-1 site and two AP-1-like elements. The Fra-1 protein is shown to lack any transactivation function, and yet it possesses oncogenic potential, as its overexpression in established rat fibroblasts resulted in anchorage-independent growth in vitro and in tumor development in athymic mice. Hence, induction of *fra-1* by c-Fos may contribute to cellular transformation elicited by deregulated c-Fos expression.

MATERIALS AND METHODS

Cell lines expressing different members of the AP-1 family. Wild-type and mutant rat *fra-1* genes were cloned as *Eco*RI-*Hin*dIII cDNA inserts of plasmid pRK7-Fra1, pRK7-Fra1-Δzip, or pRK7-Fra1-ΔDNA (see below) into the *Eco*RI and *Hin*dIII sites of the retroviral vector pMV-7, which is derived from the Moloney murine sarcoma virus (30). These retroviral plasmids were transfected by the calcium phosphate method into the packaging cell line GP+E-86 (33), and the viral supernatant of these cells was used to infect Rat-1A cells; this was followed by G418 selection. The FosER-expressing Rat-1A, PC12, and Ep-1 cell lines as well as the Rat-1A cell lines expressing v-Fos (FBR), mouse FosB, mouse c-Jun, or the hormone-binding domain of the human estrogen receptor have been previously described (2, 44, 55, 69).

Northern (RNA) blot, S1 nuclease, and RNase protection analyses. Cytoplasmic RNA was prepared from cultured cells by the method of Favaloro et al. (12), and poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography. For Northern blot analysis, either total cytoplasmic RNA (15 to 20 μg) or poly(A)$^+$ RNA (~5 μg) was separated on 1% agarose-formaldehyde gels, and this was followed by transfer of the RNA to nylon filters and hybridization with randomly primed cDNA probes as described elsewhere (8). The following cDNAs were used as probes: mouse c-*jun* (49), mouse *junB* (47), mouse *junD* (24), rat *fra-1* (9), and rat *gapdh* (13). A 600-bp cDNA fragment of the rat *fra-2* mRNA, which was amplified by reverse transcription PCR from Rat-1A cells with the primers 5′GCGGAATTCACAGCAGAAATTCCGPuGTAGATATGCC3′ and 5′GCG GTCGACTCPyTCCAGPuGGCTCCTGPyTTCACCAC3′, was cloned into the *Sal*I and *Eco*RI sites of plasmid pSP65.

S1 nuclease analysis was performed with total cytoplasmic RNA (15 μg) and 15,000 cpm of each end-labelled DNA probe as described previously (7). A 1.7-kb *Eco*RI-*Bam*HI fragment of plasmid pAγ-3′E (4) was end labelled at the *Bam*HI site in the second exon of the γ-globin gene, thus giving rise to a 209-bp S1-resistant DNA fragment upon protection by γ-globin mRNA. The *fos* S1 DNA probe was prepared as a 1.7-kb *Sca*I-*Eag*I fragment of pfos-4 (4), which was labelled at the *Eag*I site in the first exon of the mouse c-*fos* gene. This probe was protected by exogenous *fos* mRNA to yield a 196-bp S1-resistant DNA fragment (4). An 820-bp end-labelled *Sac*I-*Nar*I DNA fragment of plasmid prfra1-1.6 (see below) was end labelled at the *Nar*I site in the leader of the rat *fra-1* gene and used as an S1 probe for determining the 5′ ends of the *fra-1* mRNAs.

RNase protection analysis was carried out as previously described (60) with riboprobes that were obtained by SP6 transcription of the following plasmids. A 312-bp *Xba*I-*Hin*dIII fragment from the 3′ noncoding region of the rat *fra-1* cDNA (9), a 156-bp *Eco*RV-*Sty*I fragment of mouse S16 cDNA (61), and a 251-bp *Xho*I-*Bgl*I DNA fragment of the rat *gapdh* cDNA (13) were inserted in the antisense orientation into the polylinker of pSP64, while a 290-bp *Xba*I-*Sty*I fragment containing the 5′ sequences of mouse *fra-1* cDNA was cloned into plasmid pSP65.

TABLE 1. Oligonucleotides synthesized for PCR-based mutagenesis of *fra-1*

Oligonucleotide	Sequence
1	5′CTGGGTCCATGAGAACCCAGT3′
2	5′GCGGTAGACGATGCTTGGCACAAGGTGGAA3′
3	5′GCGGGCGCCGCTGCGACACACCC3′
4	5′GCGGTCGACACTCGCGCCTCGCAGAGTCT3′
5	5′GCGGTCGACGAACCGCAGCCGCCGACGCGG3′
6	5′CGCAGAATGTGCAGGATCCTCAGTCGCGAC3′
7	5′CTGGGGGTCCCTGAGCAGCAG3′
8	5′GCGGTCGACGCTCTTCCCTCCCCTGGCGAC3′
9	5′GCGGTCGACCCCTTCCTGGCCCCAAACGGC3′
10	5′CTCAAACTTCCAGAGATCTGCC3′
11	5′TCGACAGTGGGGCATGCATTGA3′
12	5′GCGGTCGACAGACAGCTGCCATATCCTCC3′
13	5′GCGGTCGACCTGTTTGTTCAGTAGTTGGAA3′

Cloning and sequencing of the rat *fra-1* gene. A rat genomic library constructed in the λ phage EMBL-3 (Clontech, Palo Alto, Calif.) was screened with ^{32}P-labelled rat *fra-1* cDNA, and one phage clone (12.1) containing 5 kb of *fra-1* 5′ flanking sequences within its 10.2-kb insert was isolated. The insert of this phage was subcloned into plasmid pSP64 as two separate *Bam*HI fragments with lengths of 5.5 kb and 4.7 kb (prfra1-5.5 and prfra1-4.7). The entire 5.5-kb fragment (5′ flanking sequences, exon 1, and the 5′ half of intron 1) and part of the 4.7-kb fragment (including the 3′ half of intron 1 and exon 2) were sequenced. A 1.6-kb *Sac*I fragment containing the first 710 bp of the 5′ flanking sequence, exon 1, and part of intron 1 was subcloned from phage 12.1 into the *Sac*I site of pSP64 (prfra1-1.6).

5′ End determination of the *fra-1* transcripts. The oligonucleotide 5′GAAT GAAAAGTTATTGGGCTGAACCACTGCACCGC3′ was 5′ end labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase for reverse transcription of the 5′ terminal *fra-1* mRNA sequences. The labelled oligonucleotide (10^5 cpm) was hybridized to 4 μg of poly(A)$^+$ RNA isolated from Rat-1A–FosER cells at 30°C for 15 h in 80% formamide, 0.4 M NaCl, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4), and 1 mM EDTA. After ethanol precipitation, the RNA was reverse transcribed at 37°C for 2 h in 20 μl of a buffer containing 50 μM (each) deoxynucleoside triphosphates, 50 mM Tris (pH 8.3), 8 mM MgCl, 30 mM KCl, 10 mM dithiothreitol, 40 U of RNasin, and 100 U of SuperScript reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, Md.). The cDNA products were analyzed on an 8% polyacrylamide-urea gel.

Generation of chimeric *fra-1*–β-*globin* genes. The plasmid pGlob-3 was generated by cloning a 1,780-bp *Pst*I-*Eco*RI fragment of OVEC (65) containing the promoterless rabbit β-globin gene into the polylinker of pSP64. Construct pfraβ-1 was obtained by cloning an 815-bp *Sac*I-*Nar*I fragment of the *fra-1* promoter of prfra1-1.6 upstream of the β-globin gene of pGlob3. To generate construct pfraβ-2, a 1,500-bp *Sac*I-*Eco*RI fragment of prfra1-1.6 was linked at the *Eco*RI site in the first *fra-1* intron to a 1,950-bp *Eco*RI-*Acc*I fragment, which was obtained from phage 12.1 by PCR amplification with primers 1 and 2 (Table 1). These ligated DNA fragments were cloned into the *Sac*I and *Acc*I sites of pGlob-3. As a unique *Spe*I site is present at position −170 in the *fra-1* promoter, the 5′ flanking sequences were extended in plasmid pfraβ-3 by replacing the 540-bp *Sac*I-*Spe*I fragment of pfraβ-2 with a 1.7-kb *Eco*RI-*Spe*I partial digestion fragment of prfra1-5.5 by using a *Sac*I-*Eco*RI adapter oligonucleotide. Similarly, construct pfraβ-4 was generated by replacing the same *Sac*I-*Spe*I fragment of pfraβ-2 with a 3.5-kb *Eco*RI-*Spe*I partial digestion fragment of prfra1-5.5. The AP-1 sequence in the leader of construct pfraβ-5 was mutated to a *Sal*I site by replacing the 480-bp *Nar*I-*Nru*I fragment of pfraβ-2 with a 110-bp *Nar*I-*Sal*I fragment ligated to a 270-bp *Sal*I-*Nru*I fragment, both of which were obtained from pfraβ-2 DNA by PCR amplification with primers 3 and 4 and 5 and 6, respectively (Table 1). Plasmid pfraβ-6 was derived from pfraβ-5 by replacing the 2,540-bp *Sal*I-*Acc*I fragment with a corresponding 180-bp cDNA fragment which was amplified by PCR from rat *fra-1* cDNA (9) with primers 2 and 5 (Table 1). Construct pfraβ-7 was generated by replacing the 1,120-bp *Nru*I-*Bsm*I fragment of pfraβ-2 with a 630-bp *Nru*I-*Sal*I fragment and a 430-bp *Sal*I-*Bsm*I fragment obtained by PCR amplification with primers 7 and 8 and 9 and 10, respectively (Table 1). Plasmid pfraβ-8 was created by replacing the 1,160-bp *Sph*I-*Acc*I fragment of pfraβ-2 with an 850-bp *Sph*I-*Sal*I fragment and a 310-bp *Sal*I-*Acc*I fragment, both of which were amplified by PCR from pfraβ-2 DNA with primers

11 and 12 and 2 and 13, respectively (Table 1). All constructs were verified by DNA sequencing.

Establishment of stable Rat-1A transfectants. The different *fra-1*–β-*globin* constructs (20 μg) were linearized at a unique *Bgl*I site in plasmid pSP64 and were then transfected together with the hygromycin resistance gene of plasmid pHyg (1 μg) (54) into Rat-1A–FosER cells by the calcium phosphate precipitation method. Pools of stable transfectants were obtained by selection in phenol red-free Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 1 mM glutamine, and 0.2 mg of hygromycin B per ml (Sigma).

In vitro translation and electrophoretic mobility shift assay. Mouse c-*fos* and c-*jun* cDNAs were cloned in the sense orientation downstream of the SP6 promoter of the cytomegalovirus (CMV) expression vectors pRK5 and pRK7, respectively (15a). These templates, pRK7-cJun and pRK5-cFos, were linearized with appropriate enzymes and transcribed in vitro into RNA, and both the c-*fos* and c-*jun* transcripts were then cotranslated in a rabbit reticulocyte lysate as described previously (69). Electrophoretic mobility shift analysis (EMSA) was performed by incubating 1 μl of in vitro-translated proteins for 20 min at room temperature in 20 μl containing 50 mM KCl, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 4% Ficoll, and ~10 fmol of end-labelled and reassociated AP-1 oligonucleotides. Protein-DNA complexes were analyzed by electrophoresis on 4% polyacrylamide gels in 0.25× Tris-borate-EDTA buffer.

Transient transfection assay. The different AP-1 oligonucleotides (see Fig. 6A) were cloned into the *Sal*I site of plasmid pT81luc (40), which consists of the minimal herpes simplex virus thymidine kinase promoter linked to the luciferase gene. The pT81luc plasmid is referred to as tk-luc (see Fig. 6A). The different tk-luc reporter plasmids (5 μg), the reference gene pSV2CAT (2 μg) (16), and the expression plasmids pRK7-cJun (0.5 μg) and pRK5-cFos (0.5 μg) or the empty CMV expression vector pRK7 (1 μg) were transiently transfected by the calcium phosphate coprecipitation method into RAC65 cells, a differentiation-defective subclone of the embryonal carcinoma cell line P19 (42). Two days later, the cells were washed twice with phosphate-buffered saline and resuspended in 100 μl of 250 mM Tris (pH 7.0). Following three freeze-thaw cycles and a final centrifugation step, the supernatant was used for measuring luciferase and chloramphenicol acetyltransferase activities.

Generation of mutant Fra-1, c-Fos, and Gal4 fusion proteins. A rat *fra-1* cDNA clone containing the entire coding region from nucleotides 153 to 1109 of the *fra-1* sequence (9) (GenBank accession number M19651) was isolated from a λgt10 cDNA library which was established from poly(A)$^+$ RNA of estrogen-stimulated Rat-1A–FosER cells (2). The 957-bp *Sal*I insert of this cDNA clone was recloned into pRK7 to generate pRK7-Fra1. An internal 63-bp *Pst*I fragment was deleted from the *fra-1* cDNA insert of pRK7-Fra1 to obtain pRK7-Fra1-Δzip. A short oligonucleotide (5′CTAGTTCCGGAA3′ annealed with 5′CTAG TTCCGGAA3′) was inserted into the unique *Nhe*I site of pRK7-Fra1 to generate pRK7-Fra1-ΔDNA.

Plasmid pGal4-147 was constructed by cloning the DNA-binding domain (amino acids 1 to 147) of Gal4 as a 460-bp *Hin*dIII-*Cla*I fragment of pCMV-GalER (4) into the *Hin*dIII and *Xba*I sites of pRK7 with an adaptor oligonucleotide containing a *Pst*I site (5′CGATTCTGCAGGTCGACT3′ annealed with 5′CTA GAGTCGACCTGCAGAAT3′). pGal4-cFos was obtained by inserting a 1,180-bp *Bgl*I-*Bam*HI fragment of pX-cFos (55) into the *Pst*I and *Bam*HI sites of pGal4-147 with a *Pst*I-*Bgl*I adaptor oligonucleotide (5′GTTCTCGGGTTTCA ACGCCGACTACGAGGCGTCATCCTCCCGCTGCAGTAGCGCCTCCC3′ annealed with 5′AGGCGCTACTGCAGCGGGAGGATGACGCCTCGTAG TCGGCGTTGAAACCCGAGAACTGCA3′). Plasmid pGal4-cFos-Δzip was generated by replacing the 825-bp *Bst*XI fragment of pGal4-cFos with the corresponding *Bst*XI fragment of a mutant cDNA lacking codons 179 to 206 of mouse c-*fos* (39). pGal4-Fra1 was constructed by insertion of an 840-bp *Spl*I-*Bam*HI fragment of plasmid pRK7-Fra1 into the *Pst*I and *Bam*HI sites of pGal4-147 with a *Pst*I-*Spl*I linker oligonucleotide (5′GTACCGAGACTTCGGGGAA CCGGGACCGAGTTCCGGGGCTGGCAGCGC3′ annealed with 5′GTACG CGCTGCCAGCCCCGGAACTCGGTCCCGGTTCCCCGAAGTCTC GGTACTGCA3′). Plasmid pGal4-Fra1-Δzip was generated by cloning the 777-bp *Spl*I-*Bam*HI fragment of pRK7-Fra1-Δzip into pGal4-147 with the same adaptor oligonucleotide. The junctions of all fusion genes were verified by DNA sequencing.

Soft agar and tumorigenicity assays. For the soft agar assay, 10^4 cells of stable Rat-1A transfectants constitutively expressing c-*fos*, c-*jun*, or *fra-1* genes were suspended on 35-mm-diameter petri dishes in 4 ml of 35% Difco Noble agar in Dulbecco's modified Eagle's medium containing 10% newborn calf serum. Ten days later, the cells were refed by overlaying 2 ml of 0.35% agar in the same medium, and colonies were photographed 20 days after seeding. For the tumorigenicity assay, 0.1 ml of a single cell suspension of the same cell lines (~2×10^6 cells per ml) was injected subcutaneously at four positions into the back of 2-month-old BALB/c (*nu*/*nu*) mice. Each cell line was assayed by injection into three to five mice, and tumor growth was scored 20 days after the injection.

Nucleotide sequence accession number. The DNA sequence from position −710 to position +2741 of the rat *fra-1* gene was submitted to GenBank (accession number U24154).

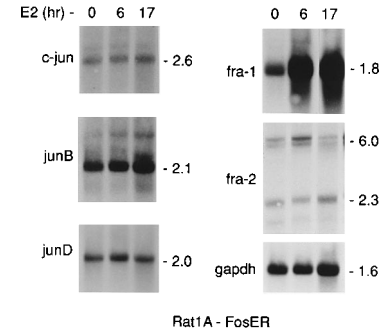

FIG. 1. Expression of different members of the AP-1 gene family in FosER-expressing fibroblasts. Rat-1A–FosER cells were either left untreated or stimulated for 6 and 17 h with 1 μM estrogen (E2). Poly(A)$^+$ RNA of these cells (~5 μg) was sequentially analyzed by Northern blot hybridization with radiolabelled c-*jun*, *junB*, *junD*, *fra-1*, and *fra-2* cDNA probes. As a control for RNA loading, the filter was rehybridized with a rat glyceraldehyde phosphate dehydrogenase (*gapdh*) cDNA probe. The sizes of the different transcripts are indicated in kilobases.

RESULTS

Expression of the *fra-1* gene is up-regulated by AP-1 activity in rat fibroblasts. Rat fibroblasts expressing FosER were analyzed by Northern blot hybridization before and after estrogen stimulation to see whether induction of Fos activity influences the expression of different members of the AP-1 gene family and hence may lead to a change in the composition of AP-1. As can be seen from Fig. 1, the levels of all three *jun* mRNAs (c-*jun*, *junB*, and *junD*) were unaffected even 17 h after estrogen activation of FosER while transcripts of the c-*fos* and *fosB* genes could not be detected at all. The *fra-2* gene gave rise to mRNAs of different sizes. The 6-kb transcripts appeared to be weakly induced 6 h after FosER activation, while the 2.3-kb mRNA remained unchanged. In contrast, transcription of the *fra-1* gene was strongly stimulated by estrogen activation of FosER as previously described (3). Hence, of all the members of the AP-1 gene family, only the *fra-1* gene was significantly up-regulated in response to increased AP-1 activity in rat fibroblasts.

fra-1 gene expression was next studied in fibroblast cell lines which ectopically expressed different members of the AP-1 gene family. As shown by Northern blot analysis, cell lines expressing c-Fos, v-Fos, and FosB contained high levels of *fra-1* mRNA in contrast to those in the parental Rat-1A cells (Fig. 2). Constitutive expression of Fra-1 and c-Jun resulted, however, in lower induction of the endogenous *fra-1* gene, indicating that *fra-1* expression is strongly stimulated only by the potent transcription factors c-Fos, v-Fos, and FosB. Overexpression of the hormone-binding domain of the estrogen receptor alone did not increase *fra-1* mRNA synthesis, indicating that the c-Fos moiety and not the hormone-binding domain of the FosER protein is responsible for *fra-1* induction (Fig. 2). Taken together, these findings indicate therefore that *fra-1* expression is activated by different members of the AP-1 gene family.

Rapid stimulation of *fra-1* transcription by FosER in different cell types. We next investigated the kinetics of *fra-1* induction in Rat-1A fibroblasts as well as in rat pheochromocytoma PC12 cells and in the mouse mammary epithelial cell line Ep-1 in order to gain insight into the cell type specificity of the *fra-1* response to FosER activation. As shown by the RNase protection experiments reported on in Fig. 3A, the *fra-1* gene is rapidly induced within 1 h of estrogen treatment in all three FosER-expressing cell lines. Interestingly, the *fra-1* mRNA

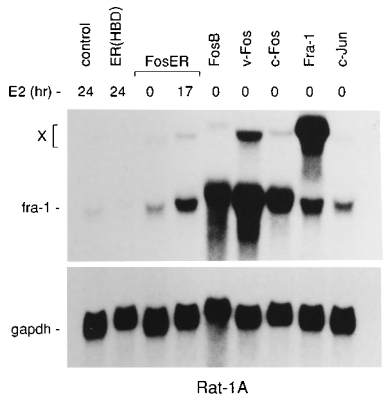

FIG. 2. Fra-1 expression is induced by different members of the AP-1 gene family. Total cytoplasmic RNA was isolated from Rat-1A cells ectopically expressing c-Fos, v-Fos (FBR), FosB, Fra-1, and c-Jun and from estrogen-treated (E2) Rat-1A cells expressing the hormone binding domain of the estrogen receptor [ER(HBD)], as well as from untreated and hormone-treated Rat-1A–FosER cells. Twenty micrograms of each RNA was used for Northern blot hybridization first with radiolabelled *fra-1* cDNA and then with a rat *gapdh* probe. Cell lines with comparable levels of exogenous c-*fos*, v-*fos*, *fosB*, *fra-1*, and c-*jun* transcripts were chosen for analysis, and expression of the exogenous protein was inferred from the observation that all selected cell lines grew in soft agar, in contrast to the parental Rat-1A fibroblasts (2). The transcripts marked X correspond to the retroviral mRNAs containing exogenous *fra-1* and v-*fos* cDNA sequences which cross-hybridize with the *fra-1* cDNA probe.

levels remained high in fibroblasts and epithelial cells upon prolonged estrogen stimulation. In contrast, induction of the *fra-1* gene was only transient in PC12 cells, as its transcript levels peaked 3 h after hormone treatment and then started to

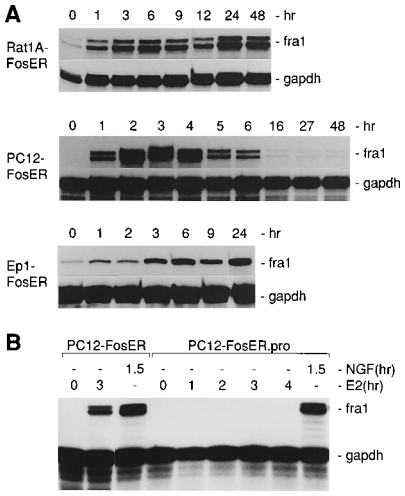

FIG. 3. Stimulation of *fra-1* expression in different cell types depends on a functional DNA-binding domain of FosER. (A) Rapid induction of *fra-1* gene expression in three different cell types. FosER-expressing cell lines derived from rat fibroblasts (Rat-1A) (55), rat pheochromocytoma cells (PC12) (69), and mouse mammary epithelial cells (Ep-1) (44) were treated with 1 μM estrogen for the indicated times. Total RNA (10 μg) of each time point was simultaneously analyzed by RNase protection with either rat *fra-1*- and *gapdh*-specific riboprobes (Rat-1A and PC12 cells) or mouse *fra-1*- and *S16*-specific riboprobes (Ep-1 cells). (B) A functional DNA-binding domain of the FosER protein is required for induction of the *fra-1* gene. The PC12 cell line expressing wild-type FosER and a previously characterized PC12 cell line expressing the mutant protein FosER.pro (69) were stimulated with estrogen (E2; 1 μM) or nerve growth factor (NGF; 50 ng/ml) for the indicated times, and total RNA was analyzed as described in the legend to panel A. The DNA-binding function of the FosER.pro mutant was inactivated by a proline insertion after amino acid 152 of the c-Fos moiety (69). The two FosER proteins were expressed to similar levels in PC12 cells (69).

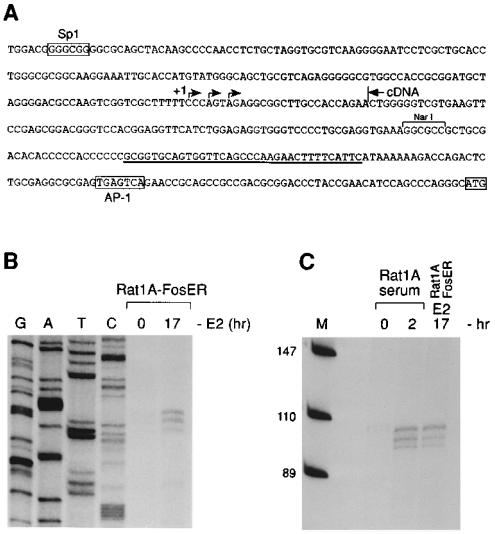

FIG. 4. Heterogeneous initiation of *fra-1* gene transcription. (A) The 5′ flanking and leader sequences of the *fra-1* gene. Arrows indicate the three start points of transcription as determined by reverse transcription and S1 nuclease analyses (panels B and C). The first start site is referred to as +1. The 5′ end of the published *fra-1* cDNA sequence (9) (GenBank accession number M19651) is shown, the translation initiation codon is boxed, the *Nar*I site used for preparing the S1 DNA probe is indicated, and the sequence which is complementary to the primer used for reverse transcription is underlined. The AP-1 site analyzed in Fig. 6 and a putative Sp1 recognition sequence are boxed. (B) Reverse transcription. Poly(A)$^+$ RNA (~4 μg) from Rat-1A–FosER fibroblasts which were untreated or stimulated for 17 h with 1 μM estrogen (E2) was used for reverse transcription with the oligonucleotide primer shown in panel A. The same oligonucleotide was used for enzymatic sequencing of the plasmid prfra1-1.6, and the products of the four sequencing reactions were coelectrophoresed with those of the reverse transcription reaction. (C) S1 nuclease analysis. Total RNA was isolated from Rat-1A cells, which were either serum starved (0) or stimulated for 2 h by the addition of 20% serum, and from Rat-1A–FosER cells, which were treated for 17 h with 1 μM estrogen (E2). This RNA (10 μg) was analyzed by S1 nuclease assay with an end-labelled *Sac*I-*Nar*I DNA fragment extending from the *Nar*I site in the leader to a *Sac*I site at position −710 in the 5′ flanking region. End-labelled pUC19 DNA digested with *Msp*I was used as a size marker (M). Sizes are given in base pairs.

decline to almost basal levels within the next 16 h (Fig. 3A). These experiments document two points. First, induction of *fra-1* expression occurs in different cell types and thus appears to be a ubiquitous response to increased AP-1 activity. Second, stimulation of *fra-1* transcription is rapid, suggesting direct regulation of the *fra-1* gene by FosER. In support of this, nuclear run-on experiments demonstrated that the transcription rate of the *fra-1* gene is increased within 1 h of hormone addition to rat fibroblasts (data not shown). Moreover, a mutant FosER protein (FosER.pro) which lacks a functional DNA-binding domain because of the insertion of a proline residue in the basic region of the c-Fos moiety (69) was unable to stimulate *fra-1* expression in PC12 cells, although up-regulation of *fra-1* by nerve growth factor treatment was still observed (Fig. 3B). Hence, an intact DNA-binding domain is required for *fra-1* induction, suggesting that FosER directly regulates the *fra-1* gene by binding to *cis* regulatory sequences.

Intragenic sequences in the 5′ region of the *fra-1* gene mediate regulation by AP-1. To identify the putative AP-1-responsive element(s) of the *fra-1* gene, we isolated the *fra-1* locus from a rat genomic library and subcloned the 5′ region of the gene. The leader and proximal promoter sequences of the *fra-1* gene are shown in Fig. 4A. The transcription initiation sites were determined by two independent methods. Both reverse transcription and S1 nuclease analyses revealed that *fra-1*

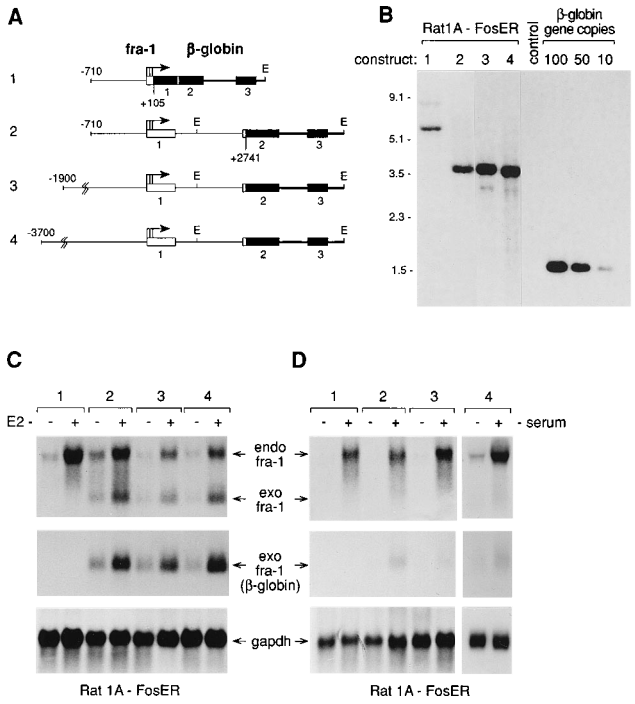

FIG. 5. Sequences in the 5′ region of the *fra-1* gene mediate induction by FosER. (A) Schematic diagram of the *fra-1* promoter constructs. The 5′ region of the *fra-1* gene was fused to the rabbit β-globin gene, and the different extents of 5′ flanking sequences used are given in base pairs relative to the first transcription start site of the *fra-1* gene. Open boxes denote *fra-1* exons, and solid boxes denote β-globin exons. For details on the construction of the chimeric genes, see Materials and Methods. E, *Eco*RI. (B) Southern blot analysis of stable Rat-1A transfectants. Cell pools derived from ~70 to 100 hygromycin-resistant colonies were generated by transfection of the chimeric genes of constructs 1 to 4 together with a neomycin resistance gene into Rat-1A–FosER cells. *Eco*RI-digested DNA of these cell pools was analyzed by Southern blot hybridization with a rabbit β-globin probe which detected an internal 3.8-kb *Eco*RI fragment for constructs 2 to 4. As construct 1 contained only a single *Eco*RI site, the same probe detected a major 6-kb fragment representing linearized DNA and several fainter bands of heterogeneous sizes corresponding to end fragments. For titration purposes, a rabbit β-globin gene fragment was coelectrophoresed in amounts corresponding to 10 to 100 copies per haploid rat genome, and rat spleen DNA was used as control DNA. The positions and sizes (in kilobases) of marker DNA are indicated to the left. (C) Fos inducibility of the *fra-1* promoter constructs. The different cell pools were grown in full serum and either left untreated (−) or stimulated by the addition of 1 μM estrogen (E2) for 17 h (+) prior to cytoplasmic RNA extraction. Total RNA (20 μg) of these pools was sequentially analyzed by Northern blot hybridization with radiolabelled rat *fra-1*, rabbit β-globin, and rat *gapdh* DNA probes. (D) Serum inducibility of the *fra-1* promoter constructs. The different cell pools were serum starved for 36 h (−) and then stimulated with 20% serum for 90 min (+). Northern blot analysis was performed as described in the legend to panel C.

transcription starts heterogeneously at three positions within a 7-bp element which is located 245 bp upstream of the translation start codon (Fig. 4B and C). Consistent with this finding, no TATA box is found in the −30 region (Fig. 4A). Moreover, computer analysis of the first 710 bp of the 5′ flanking sequences did not reveal any good match with consensus binding sites for known transcription factors except for Sp1 (Fig. 4A).

In a second step, we generated chimeric reporter genes by linking different lengths of the *fra-1* 5′ region to the rabbit β-globin gene in order to identify regulatory sequences of the *fra-1* gene in cell transfection experiments (Fig. 5A). These chimeric genes were stably transfected together with a hygromycin resistance gene into Rat-1A–FosER cells. At least two cell pools of ~70 to 100 hygromycin-resistant colonies were generated for each construct, and the analysis of one representative pool is shown in Fig. 5. Furthermore, Southern blot analysis revealed the presence of 30 to 100 copies of the transfected genes in the different cell pools (Fig. 5B). Since the transfected cells could still be transformed by FosER in a hormone-dependent manner (data not shown), we next analyzed the Fos inducibility of the endogenous and exogenous *fra-1* genes in these cells. As shown by the Northern blot analysis in Fig. 5C, the chimeric *fra-1*–β-*globin* genes of constructs 2 to 4 were as inducible by FosER activity as the endogenous *fra-1* gene, indicating that far-upstream sequences of the *fra-1* gene do not mediate the Fos effect. Instead, the Fos-responsive sequences must reside within the first 710 bp of the promoter and/or in the intragenic sequences of exon 1 and intron 1. Interestingly, no transcripts could be detected for the hybrid gene of construct 1, although the endogenous *fra-1* gene was efficiently induced in this cell pool. Construct 1 lacks most of exon 1 and all of intron 1, while it contains the same extent of promoter sequences as the Fos-inducible construct 2. We conclude, therefore, that both AP-1 inducibility and basal expression of the *fra-1* gene are dependent on intragenic sequences located between positions +105 and +2741.

All four chimeric genes were also tested for their serum inducibility. For this purpose, the different cell pools were starved and then stimulated for 90 min by serum addition. As shown by the Northern blot analysis in Fig. 5D, all of the chimeric genes responded only weakly to serum stimulation, which contrasts with the response of the endogenous *fra-1* gene. This finding suggests that the 5′ flanking sequences of the *fra-1* gene up to position −3700 lack any functionally important serum response element. The observed weak induction of the transfected *fra-1* genes is most probably mediated by the increase in AP-1 activity as a result of serum stimulation.

Identification of AP-1 binding sites in the first exon and intron of the *fra-1* gene. Sequence analysis of the 5′ region of the *fra-1* gene revealed one canonical AP-1 site (A) in the first exon and two consensus AP-1 sites (B1 and C) in the first intron (Fig. 6A). Moreover, the B1 site in the middle of intron 1 is preceded at a 14-bp interval by two AP-1-like sites (B2 and B3) which contain a cytosine instead of thymidine at the first position of the AP-1 consensus sequence. The sequences and relative positions of all putative AP-1 sites have been strictly conserved between the mouse and rat *fra-1* genes (51), suggesting the functional significance of these sites. The potential of these five sites to bind AP-1 was analyzed by EMSA with in vitro-translated c-Fos and c-Jun proteins. As can be seen in Fig. 6B, the consensus AP-1 sites A, B1, and C all bound the c-Jun–c-Fos heterodimer with an affinity that is only threefold lower than that of a reference high-affinity AP-1 site (R) (38). In contrast, the AP-1-like sites B2 and B3 interacted with the AP-1 complex very inefficiently, while no protein binding was seen with an oligonucleotide (Bm) containing C-to-T substitutions in the three B sites (Fig. 6A and B). All AP-1 sites of the *fra-1* gene were also analyzed by a transient transfection assay in embryonal carcinoma RAC65 cells which are known to contain little endogenous AP-1 activity (42). For this purpose, each AP-1 sequence was cloned as a single copy upstream of the herpes simplex virus thymidine kinase promoter which was linked to a luciferase gene. These reporter constructs were cotransfected either with CMV expression plasmids directing the synthesis of c-Jun and c-Fos or with the empty expression vector alone. As can be seen from Fig. 6C, all test genes containing one of the high-affinity AP-1 sites (A, B1, and C) were strongly (40- to 200-fold) stimulated by AP-1 activity in transfected RAC65 cells. Transcriptional stimulation was almost completely abolished by mutation of the three B sites in construct tk-luc-Bm (Fig. 6C), which is in agreement with the

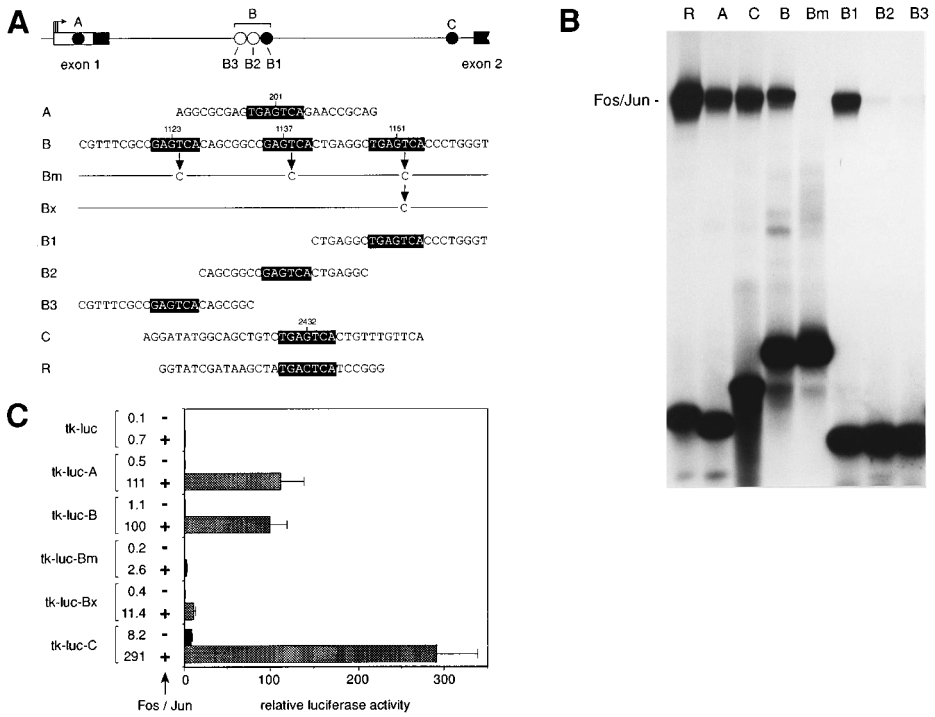

FIG. 6. Identification of AP-1-binding sites in the 5′ region of the *fra-1* gene. (A) AP-1 recognition sequences present in the 5′ region of the *fra-1* gene. A schematic diagram of exon 1 and intron 1 is shown together with the oligonucleotide sequences used for EMSA and the transient transfection assay. Leader and coding sequences are indicated by open and solid boxes, respectively. Nucleotides corresponding to the consensus AP-1 recognition sequence are highlighted by reverse type, and the positions of the AP-1 sites are given in numbers of base pairs relative to the first transcription start site. The reference AP-1 oligonucleotide R was previously described (38). (B) EMSA. DNA-binding analysis was performed with in vitro-translated AP-1 and the end-labelled oligonucleotides shown in panel A. All oligonucleotides were labelled to the same specific activity, and quantitation of the bound radioactivity (on a PhosphoImager) revealed a threefold lower affinity of AP-1 for the A, B1, and C sites compared with that for the reference high-affinity site R. (C) Transient transfection assay. Single copies of the different AP-1 oligonucleotides were cloned upstream of position −81 of the thymidine kinase promoter of a thymidine kinase-luciferase gene (40). These reporter genes were transfected together with the reference SV2CAT gene (16) and either the empty CMV expression vector pRK7 (−) or the expression plasmids pRK7-cJun and pRK5-cFos (+) into the embryonal carcinoma cell line RAC65 (42). Differences in transfection efficiencies were accounted for by normalizing the luciferase activity of the test gene to the chloramphenicol acetyltransferase activity of the reference gene. Average values of four different experiments are shown.

observation that the T-to-C substitutions interfered with AP-1 binding (Fig. 6B). Mutation of only the high-affinity site B1 in the tk-luc-Bx construct resulted in inefficient stimulation by AP-1, indicating that the intact low-affinity sites B2 and B3 are only weakly active in this assay. In summary, these DNA-binding and transfection data identified three high-affinity AP-1 sites within the intragenic region of the *fra-1* gene which mediates induction by FosER.

***fra-1* gene expression depends on sequences in the first intron.** To determine the importance of the individual AP-1-binding sites for *fra-1* gene regulation, we mutated each of these sites in the context of the *fra-1*–β-*globin* gene of construct 2 (Fig. 7A) and analyzed the expression of these mutant genes in stable transfectants of Rat-1A–FosER cells (Fig. 7B). Southern blot analysis was furthermore used to demonstrate comparable copy numbers of the transfected genes in the different cell pools (data not shown). As shown by Northern blot analysis (Fig. 7B), replacement of the AP-1 site in the *fra-1* leader sequence with a *Sal*I site did not affect expression of the *fra-1*–β-*globin* gene of construct 5, indicating that AP-1 site A is not essential for transcriptional regulation of the *fra-1* gene. However, additional deletion of the entire intron in construct 6 prevented expression of the chimeric gene altogether, even though the endogenous *fra-1* gene of this cell pool could be efficiently induced by FosER activity. Similarly, a deletion of 60 bp in the middle of intron 1 interfered with expression of the chimeric gene of construct 7. This deletion eliminated the

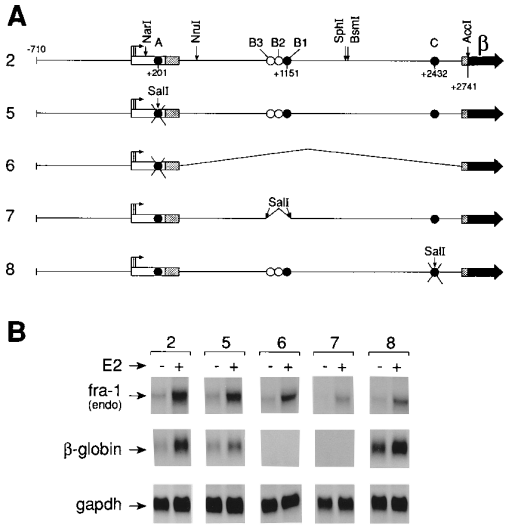

FIG. 7. Sequences in the middle of intron 1 are essential for *fra-1* gene expression. (A) Schematic diagram of the different *fra-1*–β-*globin* gene constructs. Exon 1 and intron 1 of the *fra-1* gene are shown together with the AP-1 recognition sequences identified in Fig. 6 and the restriction sites used for generating the different mutant constructs (see Materials and Methods). (B) Northern blot analysis. Pools of Rat-1A–FosER cells stably transfected with the different mutant constructs were left untreated (−) or stimulated with 1 μM estrogen (E2) for 17 h (+), and the expression of the endogenous *fra-1*, the exogenous *fra-1*–β-*globin*, and the control *gapdh* genes was analyzed as described in the legend to Fig. 5.

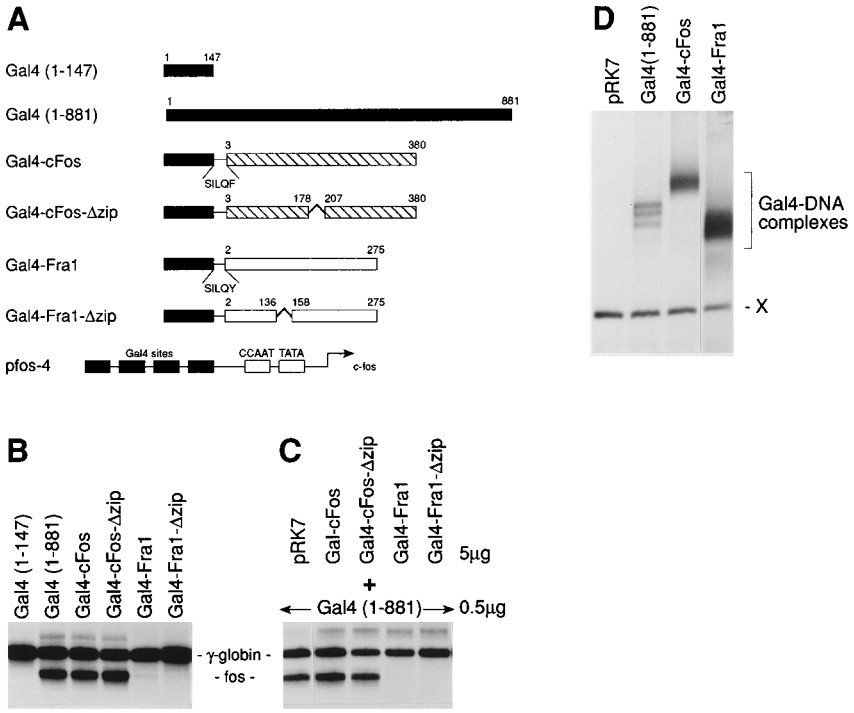

FIG. 8. Transactivation properties of chimeric Gal4-cFos and Gal4–Fra-1 proteins. (A) Schematic diagram of the Gal4 fusion proteins. The open reading frame of the mouse c-*fos* cDNA (20) or rat *fra-1* cDNA (9) was fused to the DNA-binding domain of the yeast Gal4 protein by the use of adaptor oligonucleotides which code for the amino acids indicated. The chimeric genes were cloned downstream of the CMV enhancer-promoter region of pRK7. Numbers refer to the corresponding amino acids of the respective proteins. The promoter of the Gal4-responsive gene pfos-4 (4) is shown schematically at the bottom. (B) Absence of a transactivation function in the Fra-1 protein. Expression plasmids (5 μg) directing the synthesis of the Gal4 fusion proteins shown in panel A were transiently transfected into NIH 3T3 cells together with the Gal4-responsive reporter plasmid pfos-4 (2 μg) (4) and the reference γ-globin gene pAγ-3′E (1 μg) (4). Cytoplasmic RNA was analyzed by S1 nuclease mapping with 5′-end-labelled c-*fos*- and γ-globin-specific S1 DNA probes. Plasmid pAG4 (28) was used for expression of the full-length Gal4 (1-881) protein. (C) Competition experiments. A small amount (0.5 μg) of plasmid pAG4 (28) and a 10-fold excess of the Gal4-cFos or Gal4–Fra-1 expression constructs (5 μg) were transiently transfected into NIH 3T3 cells together with reporter and reference genes, and this was followed by S1 nuclease analysis as described in the legend to panel B. pRK7 is an empty expression vector. (D) EMSA analysis. COS-7 cells were transiently transfected with 5 μg (each) of the expression plasmids pAG4, pRK7-Gal4-cFos, and pRK7-Gal4-Fra1, and nuclear extracts of the transfected cells were analyzed by EMSA, with a palindromic Gal4-binding site (4) being used as a DNA probe. Only the relevant part of the autoradiograph containing the protein-DNA complexes is shown. The Gal4-cFos and Gal–Fra-1 complexes migrated more slowly than the Gal4 (1-881) protein because of their association with endogenous Jun proteins. An endogenous DNA-binding activity of the COS-7 cells (denoted by X) served as an internal standard.

three AP-1 sites B1, B2, and B3, suggesting that they may constitute part of an intronic enhancer of the *fra-1* gene. In contrast, substitution of the high-affinity AP-1 site C with a *Sal*I sequence did not prevent expression of the chimeric gene of construct 8. We conclude therefore that transcriptional regulation of the *fra-1* gene depends on a region in the middle of intron 1 which contains AP-1-binding sites of high and low affinity.

Fra-1 lacks a transactivation function. To address the significance of AP-1-dependent regulation of *fra-1* expression, we next investigated the transactivation properties of the Fra-1 protein by fusing the entire open reading frame of Fra-1 to the DNA-binding domain (first 147 amino acids) of the *Saccharomyces cerevisiae* Gal4 protein (Fig. 8A). In addition, we deleted most of the leucine zipper region of Fra-1 to prevent dimerization and subsequent transactivation by Jun partner proteins. Equivalent fusion proteins between Gal4 and c-Fos were generated for use as controls (Fig. 8A). Expression plasmids directing the synthesis of these hybrid transcription factors were transiently transfected into NIH 3T3 cells together with a reference γ-globin gene and the Gal4-responsive reporter gene pfos-4 (Fig. 8A) (4). As shown by the S1 nuclease analysis in Fig. 8B, full-length Gal4 (1-881) and both c-Fos fusion proteins strongly stimulated transcription of a Gal4-responsive *fos* reporter gene. However, both Fra-1 fusion proteins, Gal4–Fra-1 and Gal4–Fra-1-Δzip, were unable to activate Gal4-dependent transcription, as was the polypeptide Gal4 (1-147) consisting of only the DNA-binding domain (Fig. 8B). Failed synthesis or instability of the Gal4–Fra-1 proteins was, however, ruled out in two different ways. First, all three proteins, Gal4 (1-881), Gal4-cFos, and Gal4–Fra-1, were efficiently expressed in transiently transfected COS-7 cells, as shown by the EMSA analysis reported on in Fig. 8D. Second, we performed a competition experiment by transfecting a small amount of the Gal4 (1-881) expression plasmid together with a 10-fold excess of Gal4–Fra-1 expression vectors into NIH 3T3 cells. As can be seen from Fig. 8C, expression of the Gal4–Fra-1 fusion proteins efficiently interfered with transactivation by the full-length Gal4 protein, in contrast to the situation observed with the hybrid Gal4-cFos transcription factors. Hence, the Gal4–Fra-1 proteins were synthesized in sufficient quantities within the transfected cell to prevent transcriptional stimulation by the positive regulator Gal4 (1-881). In summary, we conclude therefore that no detectable transactivation function is present in the entire Fra-1 protein.

Oncogenic potential of the Fra-1 protein. The transforming properties of members of the Fos and Jun family often correlate with their transactivation potential (1, 68), and hence the Fra-1 protein would be expected to lack any oncogenic potential. To test this hypothesis, we have used retroviral infection to

generate stable Rat-1A cell lines that overexpressed wild-type or mutant *fra-1* genes. The dimerization function of the Fra-1 protein was inactivated by partial deletion of the leucine zipper in Fra1-Δzip, while the DNA-binding potential of Fra-1 was abolished by insertion of four amino acids into the basic region in Fra1-ΔDNA. Cell lines which expressed similar levels of exogenous *fra-1* mRNA as shown by RNase protection analysis (Fig. 9B) were examined for morphological transformation, growth in soft agar, and tumor formation in nude (*nu*/*nu*) mice (Fig. 9A). Overexpression of neither wild-type nor mutant Fra-1 proteins was able to significantly alter the morphological appearance of Rat-1A cells. Similarly, overexpression of c-Jun did not morphologically transform rat fibroblasts, quite in contrast to deregulated expression of the c-Fos protein (Fig. 9A). Interestingly, both Fra-1- and c-Jun-expressing cell lines grew in soft agar, although slightly less well than c-Fos-expressing Rat-1A cells. Moreover, nude mice injected with the Fra-1-expressing cell clone developed tumors as efficiently as mice injected with c-Fos-expressing cells. However, cell lines overexpressing mutant Fra-1 proteins (Fra1-ΔDNA and Fra1-Δzip) were able neither to grow in soft agar nor to elicit tumor formation in nude mice (Fig. 9A). Hence, the dimerization function of the leucine zipper and the DNA-binding activity of the basic region of the Fra-1 protein are both required for anchorage-independent growth and tumorigenicity of Fra-1-expressing fibroblasts. Together, these data clearly demonstrate that Fra-1 has oncogenic potential despite lacking a transactivation function.

DISCUSSION

Using transcriptional and posttranslational Fos induction systems, we have previously demonstrated that the *fra-1* gene is up-regulated in response to elevated c-Fos levels in rat fibroblasts (3, 4). This observation has subsequently been confirmed by other groups (34, 52). Here we report that the *fra-1* gene is induced not only by ectopic expression of c-Fos but also by overexpression of other members of the AP-1 family (v-Fos, FosB, Fra-1, and c-Jun). Hence, the *fra-1* gene is under positive control by AP-1 activity. Furthermore, up-regulation of *fra-1* expression by AP-1 is not specific for fibroblasts but represents a more general phenomenon, as it was also observed in cell lines of neuronal (PC12) and epithelial (Ep-1) origins. In support of this, the *fra-1* gene is also expressed at elevated levels in cell lines derived from osteosarcoma and cartilage tumors of c-*fos* transgenic mice (19, 63). Recent loss-of-function experiments have furthermore corroborated an important role of AP-1 in the regulation of the *fra-1* gene, showing that serum stimulation of *fra-1* transcription is considerably reduced in fibroblasts derived from mice lacking either c-*fos* (5) or c-*jun* (51).

Transcriptional regulation of the *fra-1* gene. Transcription of the rat *fra-1* gene is initiated at three sites within a 7-bp element located 245 bp upstream of the translation start codon. Heterogeneous initiation is consistent with the absence of a TATA box in this region. The three start sites were mapped by two independent methods and agree well with the previous cloning of *fra-1* cDNA resulting in the isolation of 225 bp of 5′ nontranslated sequences (9) (Fig. 4A). By RNase protection analysis, we have recently identified additional minor transcription initiation sites that are located about 50 bp upstream of the translation start codon (17). Transcription initiation in this proximal position has also been described for the human *fra-1* gene (58).

Direct regulation of the *fra-1* gene by AP-1 was suggested by the rapid induction kinetics in response to FosER activation and by the requirement for an intact DNA-binding domain in the c-Fos moiety of FosER. Transfection experiments with chimeric *fra-1*–β-*globin* genes demonstrated that transcriptional stimulation by FosER is mediated by intragenic sequences located between positions +105 and +2741 of the *fra-1* gene. This extended region contains three AP-1 consensus sequences which proved to be functional in protein-DNA binding as well as in transactivation assays. However, mutation of these sites in the context of the *fra-1* gene revealed that only the high-affinity AP-1 site in the middle of intron 1 is located in a regulatory region that proved to be essential for *fra-1* expression. This AP-1 site is flanked by two AP-1-like elements whose sequences and relative positions have been completely conserved between the rat and mouse *fra-1* genes (51). It is therefore conceivable that all three binding sites constitute part of an intronic enhancer which controls both basal and induced transcription of the *fra-1* gene. In this context, it is interesting that AP-1 regulatory sequences in an intron position have previously been shown to confer AP-1 responsiveness to the genes coding for cytokeratin 18 (41), nerve growth factor (21), and α_{2u}-globulin (59).

The kinetics of *fra-1* induction by FosER differ in distinct cell types, suggesting that the cell-type-specific environment modulates AP-1-dependent regulation of this gene. In particular, *fra-1* induction is only transient in PC12 cells in spite of the presence of sustained FosER activity. This behavior of the *fra-1* gene is reminiscent of that of another Fos-responsive gene, the ornithine decarboxylase gene, in PC12 cells (69). Prolonged activation of FosER is known to induce PC12 cells to develop an epithelial cell-like morphology (69), suggesting that an altered regulatory environment may be responsible for the down-modulation of *fra-1* and ornithine decarboxylase gene expression in this cell type.

fra-1, like other immediate-early genes, is a nuclear target of intracellular signaling which is initiated at the cell surface by the interaction of receptors with serum growth factors (9). Sequences in the 5′ region of *fra-1* (from positions −3700 to +2741) are, however, not sufficient to confer proper serum regulation to chimeric *fra-1*–β-*globin* genes. In agreement with this finding, no consensus serum response element could be identified in these DNA sequences by computer search. The weak serum inducibility observed with these constructs results most likely from an increase in AP-1 activity in serum-stimulated fibroblasts. In this context, it is worth noting that serum regulation of the immediate-early gene *JE* is mediated by a novel 7-nucleotide motif (TTTTGTA) which is present in the 3′ noncoding region. This motif, referred to as the immediate response box, cooperates with an enhancer in the 5′ region to bring about full serum stimulation of the *JE* gene (14). Interestingly, the *fra-1* gene also contains an immediate response box motif in its 3′ noncoding sequences (14). Moreover, the serum inducibility of *fra-1* is considerably reduced in fibroblasts lacking either c-*fos* (5) or c-*jun* (51). Together, these observations suggest that both the immediate response box motif in the 3′ noncoding region and the AP-1 responsive enhancer in the first intron may be required for full serum stimulation of the *fra-1* gene.

Cross-talk among different members of the AP-1 family. Potent transactivation functions have been identified in the C-terminal regions of c-Fos (56) and FosB (67) by fusing the corresponding sequences to the DNA-binding domain of Gal4. The same approach applied to the Fra-1 protein has recently demonstrated that Fra-1 lacks C-terminal transactivating sequences (68). However, both c-Fos and FosB also contain an N-terminal transactivation domain which shows considerable similarity to a corresponding sequence of the Fra-1 protein (27,

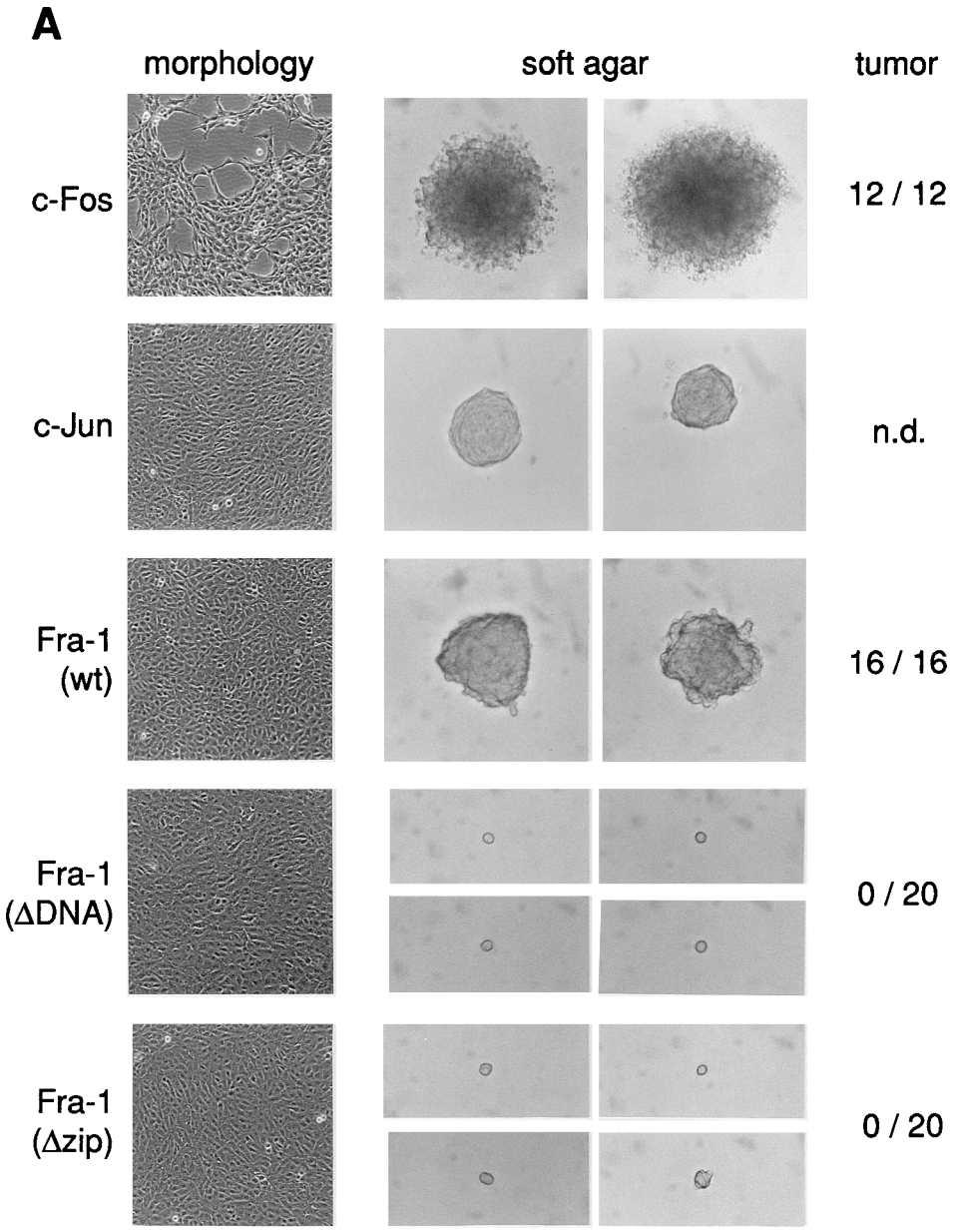

FIG. 9. Oncogenic properties of the Fra-1 protein. (A) Morphology. Stable Rat-1A cell lines overexpressing the c-*fos*, c-*jun*, or *fra-1* gene were seeded at the same low density and photographed at confluence. Soft agar assay. The same cell lines were analyzed for anchorage-independent growth. Representative colonies were photographed 20 days after seeding. Tumor formation. The different Rat-1A cell lines (2×10^5 cells) were injected into *nu*/*nu* mice, and tumor formation per injection site (ratios at right) was scored 20 days later. The Rat-1A cell lines expressing c-Fos and wild-type (wt) Fra-1 gave rise to tumors with average weights of 0.3 and 1.1 g, respectively. The protein Fra1-ΔDNA contains an insertion of four amino acids (VPEL after amino acid 119) in its basic region, while Fra1-Δzip has the same leucine zipper deletion as Gal4-Fra1-Δzip (Fig. 8A). Both proteins, Fra1-ΔDNA and Fra1-Δzip, are unable to form DNA-binding AP-1 complexes, as shown by EMSA analysis (data not shown). n.d., not determined. (B) Retroviral gene expression. The exogenous c-*fos*, c-*jun*, and *fra-1* genes were all transcribed from the Moloney sarcoma virus long terminal repeat promoter of pMV-7 (30), and hence their transcript levels could be directly compared by RNase protection analysis with a riboprobe derived from the common retroviral leader sequences.

67). Analysis of the full-length Fra-1 protein in the Gal4 fusion assay has now demonstrated that the N-terminal sequences of Fra-1 also lack a transactivation function despite the observed sequence homology. In agreement with this finding, Suzuki et al. reported that Fra-1, like Fra-2, is able to inhibit AP-1-dependent transactivation by c-Fos and c-Jun in transiently transfected embryonal carcinoma cells (57). Hence, Fra-1 could be considered to be a negative regulator of AP-1 activity. Fra-1 is expressed with delayed and protracted kinetics relative to those of the potent transcriptional activators c-Fos and FosB in serum-stimulated fibroblasts (9, 31). Consequently, Fra-1 is a predominant Fos protein only at later times during serum stimulation (31). One function of Fra-1 could therefore be to limit the transcriptional activity of c-Fos and FosB during the transition from G_0 to G_1 by sequestering Jun partner proteins into less active AP-1 complexes. A similar function has been assigned to the FosB2 protein (also known as ΔFosB, FosB-S, and FosB/SF), which, as a result of alternative splicing, lacks the C-terminal transactivation domain of full-length FosB (36, 37, 70). Transcriptional cross-regulation among members of the *fos* gene family is yet a further mechanism that results in the down-modulation of AP-1 activity following serum stimulation. As shown here, c-Fos and FosB are potent activators of *fra-1* transcription, while c-Fos, FosB, and Fra-1 are able to repress transcription of the c-*fos* gene (15, 36, 50, 66).

Fra-1 and Fra-2 are both expressed at significant levels in cycling cells, in contrast to c-Fos and FosB (31). Moreover, injection of neutralizing anti-Fra-1 antibodies into exponentially growing fibroblasts was shown to partially block DNA synthesis, suggesting that Fra-1 may exert an important function during cell cycle progression (31). It is, however, unlikely that Fra-1 acts solely as a negative regulator of AP-1 activity in cycling cells for the following reasons. First, Jun as well as Fos proteins contribute to the transcriptional activity of the heterodimer (23). Second, binding of Fra-1 to Jun proteins is known to stabilize the AP-1 complex in a manner similar to that of c-Fos, thus resulting in increased DNA binding of the heterodimer (10, 48). As a consequence, Fra-1 forces Jun proteins into stable DNA-binding complexes and may thus activate AP-1-responsive genes by tethering the Jun transactivation function to their regulatory sequences. This hypothesis could explain why ectopic expression of Fra-1 results in upregulation of its own gene (Fig. 2).

Oncogenic potential of Fra-1 in the absence of a potent transactivation function. Cellular transformation by members of the Fos family is considered to be dependent on the dimerization function of the leucine zipper, the DNA-binding activity of the basic region, and the presence of a potent transactivation domain (27, 68). Consistent with this notion, no transforming potential could be assigned to Fra-1 by focus formation assays (68). Moreover, we could also not detect any morphological transformation of established fibroblasts which constitutively expressed Fra-1. However, these fibroblasts were capable of anchorage-independent growth in vitro and tumor formation in nude mice, which is reminiscent of transformation by the FosB2 protein. In analogy to the situation with Fra-1, constitutive expression of FosB2 neither induces morphological changes nor stimulates focus formation in rat fibroblasts (32, 36, 70). FosB2-expressing Rat-1A cells grow, however, to higher saturation density, display anchorage-independent growth, and generate tumors with a short latency period in nude mice (32). It appears, therefore, that Fra-1 and FosB2 have similar oncogenic potentials in the absence of a transactivation function.

In summary, we have demonstrated that AP-1-dependent regulation of the *fra-1* gene is mediated by intragenic sequences. As the Fra-1 protein lacks a transactivation domain, the AP-1-dependent increase in Fra-1 levels may be used to limit the activity of the potent transcription factors c-Fos and FosB during growth factor stimulation of cells. Moreover, as Fra-1 is an oncoprotein, its increased expression may also contribute to cellular transformation elicited by deregulated expression of other members of the AP-1 family. Clearly, the transcriptional regulation of *fra-1* is unique among members of the *fos* gene family, as only *fra-1* is subject to positive control by AP-1 activity in rat fibroblasts.

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