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Cell-Specific Regulation of Oncogene-Responsive Sequences of the c-*fos* Promoter

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We have identified oncogene-responsive sequences in the human c-*fos* promoter that mediate induction of transcription by several nonnuclear oncoproteins and the tumor promoter TPA. These sequences are regulated in a cell-specific manner. (i) In NIH 3T3 cells, the CArG box of the c-*fos* promoter is sufficient to mediate activation by oncogenes. (ii) In contrast, in HeLa cells, additional flanking sequences are also required, including the outer arm of the serum response element and the FAP site. We also show that the serum response factor, which binds to the CArG box, activates transcription in vivo in NIH 3T3 cells but not in HeLa cells. Finally, we present evidence that the intracellular level of the c-Fos protein could be a major determinant of cell-specific regulation of these oncogene-responsive elements of the c-*fos* promoter.

The c-*fos* proto-oncogene is the best-characterized member of the early-response genes. Its transcription is induced rapidly, albeit transiently, by serum components, growth factors, and a number of oncogenes (15, 27, 29, 31, 34). A key role in its regulation is played by the serum response element (SRE), which binds several different proteins (reviewed in references 24 and 34; see Fig. 1A). The inner core, or CArG box, binds the serum response factor (SRF) (23, 33). Mutations in the CArG box that abolish SRF binding eliminate induction by serum, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and several growth factors in different cell lines (5, 6, 10, 30, 32). Although this sequence has been found to be necessary and sufficient for induction (25), there is evidence that other factors are also involved. Mutations in the outer arm of the SRE that prevent binding of the factor $p62^{TCF}$ impair serum and TPA inducibility (8, 29). The *fos* gene is also negatively regulated by its own product through the CArG box (7, 17–19, 25, 28).

An AP1-like site (FAP), located a few nucleotides downstream from the SRE, is also serum responsive. It binds members of the AP1 and CREB families, but only in the absence of SRF because of steric hindrance (5, 35). Binding of Fos to the FAP site is not involved in autorepression, since Fos mutated in its DNA-binding domain can still repress (17, 19).

We show here that the sequences of the c-*fos* promoter that mediate induction of transcription by several nonnuclear oncoproteins differ between cell types. In NIH 3T3 cells, the CArG box is sufficient, whereas in HeLa cells the outer arm of the SRE is also required. Interestingly, SRF, when introduced alone, activates transcription in NIH 3T3 cells but not in HeLa cells. In HeLa cells, another combination of sites, the outer arm and FAP, constitute an alternative *ras* responsive unit. Finally, we present evidence that the intracellular level of the Fos protein could be a major determinant of this cell-specific regulation.

MATERIALS AND METHODS

Construction of recombinants. Reporter recombinants were constructed by inserting the oligonucleotides depicted

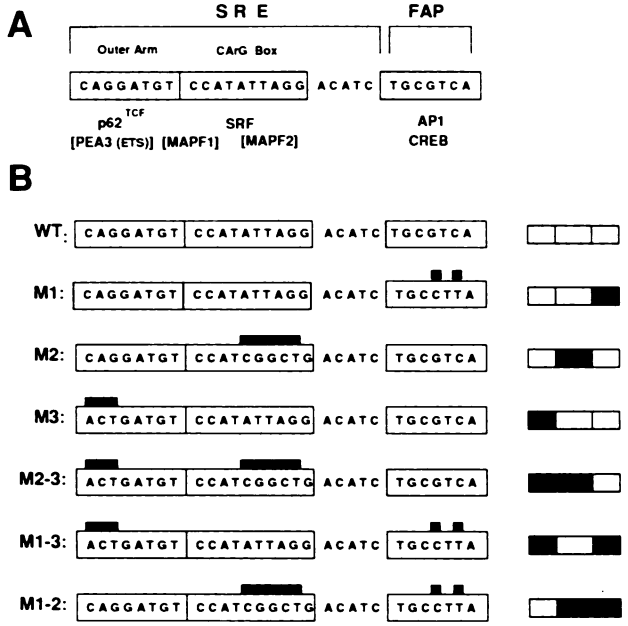

FIG. 1. (A) Sequence of the human c-*fos* promoter between nucleotides −317 and −288. The SRE and the *fos* AP1 (FAP) site are illustrated. The SRE is divided into the CArG box, which binds the SRF, and the outer arm, which binds $p62^{TCF}$ (when complexed to SRF). FAP binds members of the AP1 and CREB families. Other factors that interact with the *fos* promoter are represented between brackets and below their binding sequences (PEA3 [11]; MAPF1 and MAPF2 [26, 36]). (B) Sequence of oligonucleotides used to construct reporter recombinants. They encompass the human c-*fos* promoter between nucleotides −317 and −288. The outer arm, the CArG box, and the FAP regions are boxed. WT indicates wild type. Mutations that abolish the binding of AP1 (1), SRF (29), or $p62^{TCF}$ (29) were introduced into recombinants M1, M2, and M3, respectively. Pairs of mutations were introduced into M2-3, M1-3, and M1-2. A mutated base is indicated by a black box over the base. The schematic structure of each recombinant is represented by three boxes corresponding to the three main regions described in panel A. White boxes represent the wild-type sequences and black boxes represent the mutated sequences.

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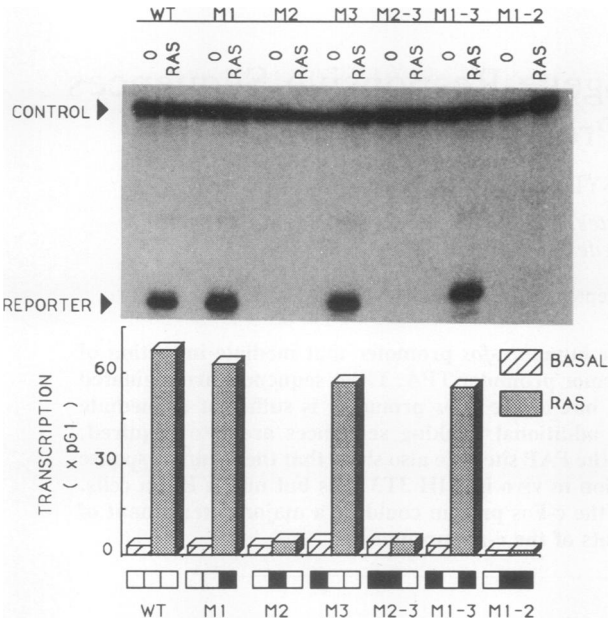

FIG. 2. Effect of expression of the Ha-*ras* oncogene on transcription activity of wild-type (WT) and mutated recombinants in NIH 3T3 cells. The schematic structure of each reporter recombinant is shown. Subconfluent NIH 3T3 cells were transfected with 5 μg of the indicated reporters, 1 μg of the internal control plasmid (pβCBX2), and 12 μg of an Ha-*ras* expression vector. After 36 h in culture in low serum (0.5% FCS), total RNA was analyzed by quantitative S1 nuclease mapping for RNA initiated from the β-globin promoter of the reporters (reporter bands) and the conalbumin promoter of the control recombinants (control bands). The ratio of specific transcription from the insert containing reporters to that of the parent vector is represented by a histogram. Transcription values are the averages from at least two experiments and are corrected for variation in pG1 expression with Ha-*ras*. The pG1 transcription level is considered equal to 1. Lanes are with (RAS) and without (0) added Ha-*ras*.

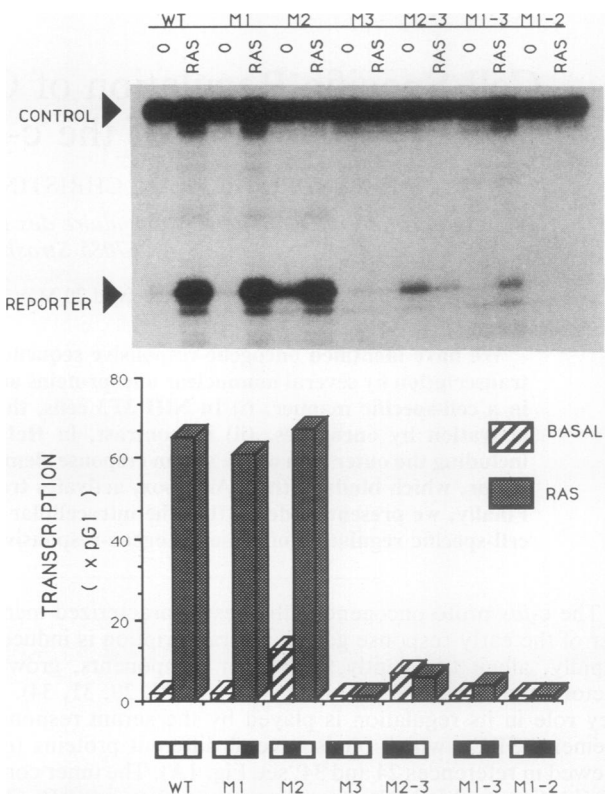

FIG. 3. Effect of expression of the Ha-*ras* oncogene on transcription activity of wild-type and mutated recombinants in HeLa cells. The schematic structure of each reporter recombinant is shown. Subconfluent HeLa cells were transfected with 5 μg of the indicated reporters, 1 μg of the internal control plasmid (pβCBX2), and 12 μg of a Ha-*ras* expression vector. After 36 h in culture in low serum (0.5% FCS), total RNA was analyzed by quantitative S1 nuclease mapping for RNA initiated from the β-globin promoter of the reporters (reporter bands) and the conalbumin promoter of the control recombinants (control bands). The ratio of specific transcription from the insert containing reporters to that of the parent vector is represented by a histogram. Transcription values are the averages from at least two experiments and are corrected for variation in pG1 expression with Ha-*ras*. The pG1 transcription level is considered equal to 1. Lanes are with (RAS) and without (0) added Ha-*ras*.

in Fig. 1B (containing linkers for *Xho*I and *Sal*I and annealed previously with their corresponding complementary oligonucleotides) in the *Xho*I site of the pG1 expression vector in the 5′ to 3′ orientation. Sequences were verified by the Sanger method. The SRF cDNA which was cloned in the pSG5 expression vector (9) is under the control of the simian virus 40 early promoter.

Transfections. Transfections were performed as follows. (i) For LMTK$^-$ cells, subconfluent 9-cm-diameter plates were incubated for 30 to 60 min with DEAE-dextran (0.5 mg/ml) containing 1 μg of reporter recombinant, 5 μg of an oncogene expression vector, and 1 μg of the internal control plasmid pβCBX2. Cells were washed and incubated for 3 h with 100 μM cloroquine phosphate in culture medium supplemented with 10% fetal calf serum (FCS). After being washed, cells were incubated in low-serum (0.5% FCS) culture medium until they were harvested 36 h later. TPA (300 ng/ml) was added 12 h posttransfection for 24 h. (ii) For NIH MT-FOS, NIH 3T3, and HeLa cells, subconfluent cells were transfected by the calcium phosphate method with 5 μg of a reporter recombinant, 1 μg of the internal control plasmid (pβCBX2), and 12 μg of an oncogene expression vector. They were incubated with the calcium phosphate precipitate in culture medium containing 10% FCS for 16 to 20 h and then washed and incubated in low-serum (0.5% FCS) culture medium for 36 h. Cadmium chloride (6 μM) was added, where indicated (see Fig. 5), during the 20-h period prior to the harvesting of the cells. Transfections were repeated at least twice for each experimental point, and variability did not exceed 20%.

S1 nuclease analysis. The structure of oncogene expression vectors was described previously (38). The probe used in quantitative S1 mapping was an 80-mer oligonucleotide containing 59 nucleotides complementary to the −9 to +50 region of the rabbit β-globin gene and 21 noncomplementary nucleotides. The first 50 nucleotides of the β-globin mRNA transcribed from the reporter recombinants hybridize specifically with this probe, giving, after digestion with S1 nuclease, a 50-base band. The same probe also hybridizes with nucleotides −9 to +50 of the RNA transcribed from the control recombinant pβCBX2 (which contains the conalbumin promoter), giving a 59-base band. An excess of this probe (10^5 cpm; 10 fmol) was hybridized overnight at 65°C with total RNA (up to 50 μg) in 10 μl of 0.04 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES, pH 7)–0.4 M

TABLE 1. Activation of transcription by oncoproteins and TPA[a]

Oncogene or treatment	Activation relative to pG1					
	LMTK cells			HeLa cells		
	M1	M1-3	M1-2	M1	M1-3	M1-2
Basal	3	1	1	3	2	1
Ha-*ras*	57	3	2	60	5	1
v-*src*	48	2	1	22	7	1
Py-*mt*	62	2	1	37	5	1
v-*mos*	45	2	2	28	1	1
v-*raf*	16	1	1	60	2	1
TPA[b]	13	1	1			

[a] Transfections and S1 mapping were performed as indicated in the legend to Fig. 3. The pG1 transcription level is considered equal to 1.

[b] TPA was added at a concentration of 300 ng/ml during the 12- to 36-h posttransfection period.

NaCl–1 mM EDTA, and S1 mapping was performed as previously described (38). The specific bands were quantitated by scanning adequately exposed autoradiograms.

RESULTS

In NIH 3T3 cells, the CArG box is necessary and sufficient to mediate transcription activation by the Ha-*ras* oncogene. Transcriptional activation by *ras* of the human c-*fos* promoter was investigated by using recombinants containing wild-type and mutated elements (from nucleotides −317 to −288; Fig. 1A) in the expression vector pG1 (Fig. 1B). Mutations that abolish the binding of AP1 (M1 [1]), SRF (M2 [29]), and $p62^{TCF}$ (M3 [29]) were introduced individually or in pairs (M2-3, M1-3, and M1-2). These recombinants were transiently transfected in NIH 3T3 cells, together with control or *ras* expression vectors. Basal and *ras*-induced transcription was measured by S1 nuclease mapping (Fig. 2). The wild type is strongly induced by *ras* expression (Fig. 2, WT). Mutations in the FAP site and in the outer arm, either individually (M1 and M3, respectively) or in combination (M1-3), do not affect activation by *ras*. In contrast, mutations in the CArG box (M2, M2-3, and M1-2) completely abolish *ras* induction. Indeed, the CArG box alone is sufficient to mediate transcription activation by *ras* (Fig. 2, M1-3).

In HeLa cells, sequences besides the CArG box are critical for transcription activation by the Ha-*ras* oncogene. In HeLa cells, transcription of the wild-type recombinant is strongly induced by Ha-*ras* expression (Fig. 3, WT). However, neither the CArG box nor the other motifs alone respond to *ras* expression (Fig. 3; M2-3, M1-3, and M1-2). The outer arm is essential for the activation of transcription by *ras*, in combination either with the CArG box or with the FAP site (Fig. 3, M1 and M2, respectively). However, it is not sufficient (Fig. 3, M3), showing that there is cooperation

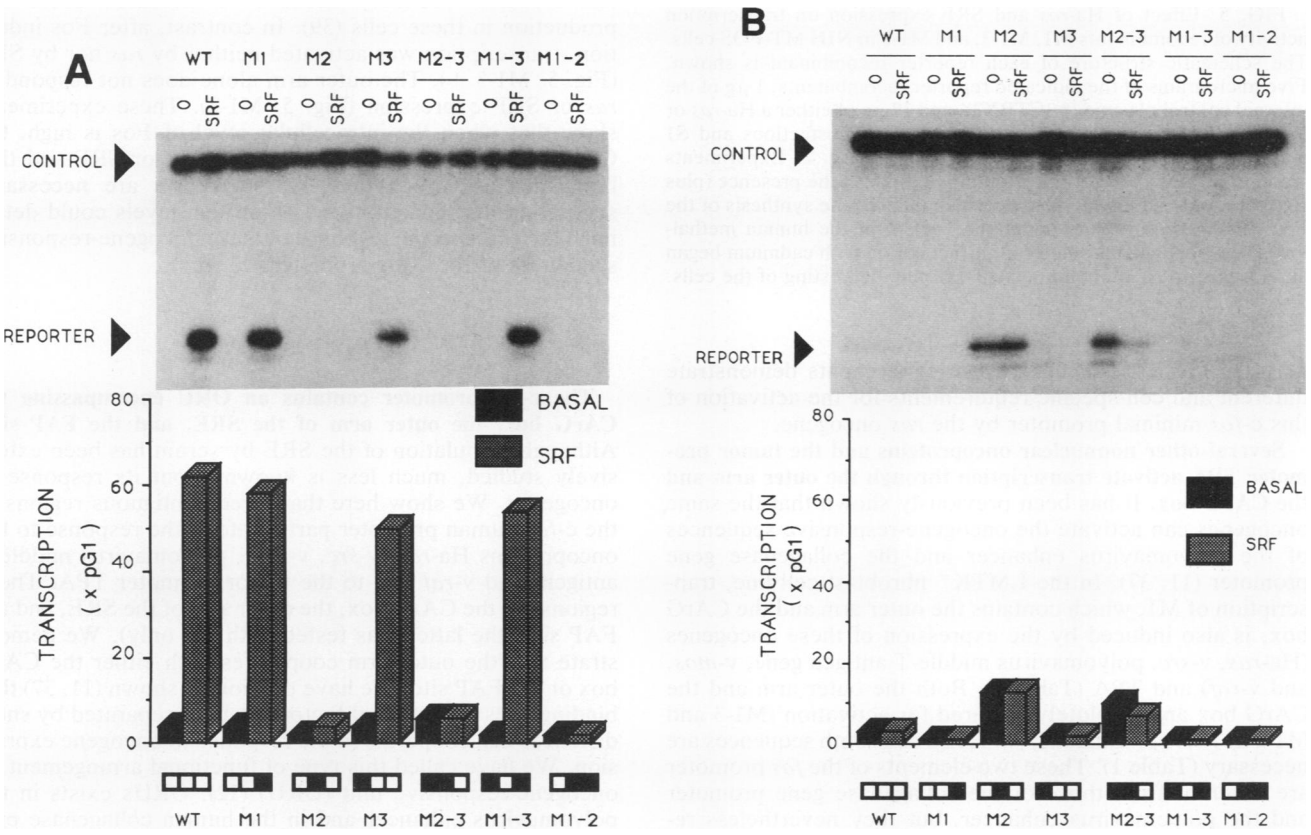

FIG. 4. Effect of expression of SRF on transcription activity of wild-type and mutated recombinants in NIH 3T3 cells (A) and in HeLa cells (B). The schematic structure of each reporter recombinant is shown. Five micrograms of reporter recombinants, 1 μg of control vector, and 12 μg of the SRF expression vector were transfected in NIH 3T3 and HeLa cells. Transfections and S1 mapping were performed as indicated in the legends to Fig. 2 and 3. Lanes are with (SRF) and without (0) added SRF.

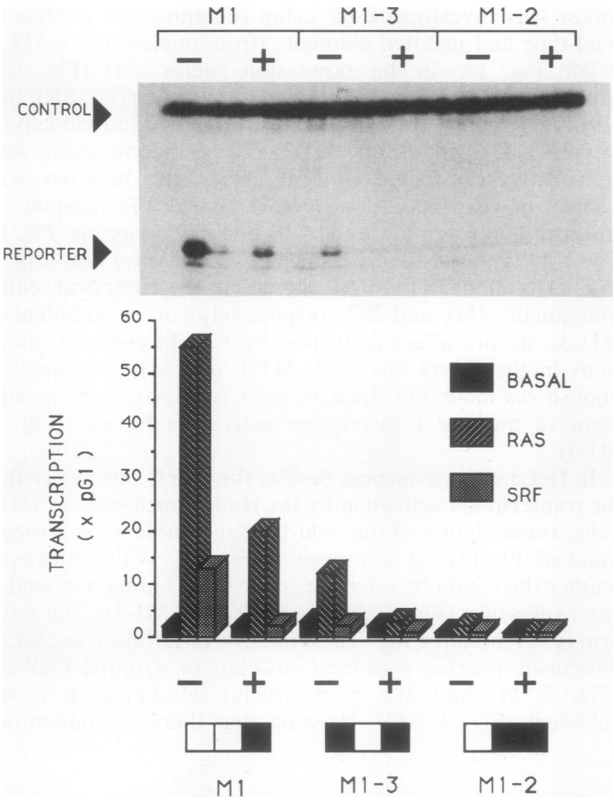

FIG. 5. Effect of Ha-*ras* and SRF expression on transcription activity of recombinants M1, M1-3, and M1-2 in NIH MT-FOS cells. The schematic structure of each reporter recombinant is shown. Five micrograms of the indicated reporter recombinants, 1 μg of the internal control plasmid (pβCTBX2), and 12 μg of either a Ha-*ras* or a SRF expression vector were transfected. Transfections and S1 mapping were as described in the legend to Fig. 2. Experiments were performed in the absence (minus sign) or in the presence (plus sign) of 6 μM cadmium chloride, which induces the synthesis of the Fos protein from a gene under the control of the human methallothionein II A gene promoter (38). Incubation with cadmium began at 16 h posttransfection and ended with the harvesting of the cells.

between these sequences. These experiments demonstrate different and cell-specific requirements for the activation of this c-*fos* minimal promoter by the *ras* oncogene.

Several other nonnuclear oncoproteins and the tumor promoter TPA activate transcription through the outer arm and the CArG box. It has been previously shown that the same oncogenes can activate the oncogene-responsive sequences of the polyomavirus enhancer and the collagenase gene promoter (11, 37). In the LMTK$^-$ fibroblast cell line, transcription of M1, which contains the outer arm and the CArG box, is also induced by the expression of these oncogenes (Ha-*ras*, v-*src*, polyomavirus middle-T antigen gene, v-*mos*, and v-*raf*) and TPA (Table 1). Both the outer arm and the CArG box are absolutely required for activation (M1-3 and M1-2 in Table 1). Similarly, in HeLa cells both sequences are necessary (Table 1). These two elements of the *fos* promoter are different from those of the collagenase gene promoter and the polyomavirus enhancer, but they nevertheless respond to the same oncogenes.

The SRF is a cell line-specific transcriptional activator. We expressed SRF in vivo from a pSG5-based (9) expression vector containing the SRF cDNA under the control of the simian virus 40 early promoter. This vector efficiently expressed SRF, as shown by gel retardation experiments with extracts of transfected COS cells (results not shown). In NIH 3T3 cells, transcription of the wild-type construct is efficiently stimulated by SRF expression (Fig. 4A, WT). Only mutations in the CArG box abolish activation by SRF (Fig. 4A; M2, M2-3, and M1-2). In contrast, in HeLa cells (Fig. 4B), SRF does not activate any of the reporters, even though different quantities (from 0.25 to 20 μg) of SRF expression vector were used (data not shown). The pSG5 expression vector is functional in HeLa cells, as shown by the results for pSG5-*src* (v-*src*) in Table 1 and by other work from this laboratory. Thus, SRF is a cell line-specific transcription factor.

Intracellular levels of Fos contribute to cell line-specific regulation of the oncogene-responsive sequences of the c-*fos* promoter. Synthesis of the Fos protein can be induced by cadmium in NIH MT-FOS cells, which contain an exogenously added c-*fos* gene under the control of the human methallothionein II A gene promoter (39). NIH MT-FOS cells were transiently transfected with the *fos* reporter recombinants and expression vectors for *ras* and SRF. In noninduced cells, both *ras* and SRF activated transcription of M1 (Fig. 5, M1 −), as they did in the parental NIH 3T3 cells. Fos synthesis diminished activation by *ras* and completely abolished stimulation by SRF (Fig. 5, M1 +). The CArG box was sufficient for a low level of activation by *ras* but not by SRF (Fig. 5, M1-3 −). In noninduced conditions, MT-FOS cells have a behavior intermediate between that of NIH 3T3 cells and that of induced MT-FOS cells (Fig. 2, 4A, and 5), most probably because of increased basal Fos production in these cells (39). In contrast, after Fos induction, transcription was activated neither by *ras* nor by SRF (Fig. 5, M1-3 +). The outer arm alone does not respond to *ras* or SRF expression (Fig. 5, M1-2). These experiments show that when the intracellular level of Fos is high, the CArG box alone does not respond to *ras* or SRF and that both the CArG box and the outer arm are necessary, suggesting that constitutive Fos protein levels could determine the cell-specific response of these oncogene-responsive sequences of the c-*fos* promoter.

DISCUSSION

The c-*fos* promoter contains an ORU encompassing the CArG box, the outer arm of the SRE, and the FAP site. Although stimulation of the SRE by serum has been extensively studied, much less is known about its response to oncogenes. We show here that three contiguous regions of the c-*fos* human promoter participate in the response to the oncoproteins Ha-*ras*, v-*src*, v-*mos*, polyomavirus middle-T antigen, and v-*raf* and to the tumor promoter TPA. These regions are the CArG box, the outer arm of the SRE, and the FAP site (the latter was tested with *ras* only). We demonstrate that the outer arm cooperates with either the CArG box or the FAP site. We have previously shown (11, 37) that binding sites for different factors that are separated by short distances can cooperate in the response to oncogene expression. We have called this type of functional arrangement an oncogene-responsive unit (ORU) (12). ORUs exists in the polyomavirus enhancer and in the human collagenase promoter (11, 37). The two subregions of the SRE and the FAP site could also be considered to be ORUs. These ORUs from different promoters contain distinct sequences; nevertheless, the same oncogenes activate transcription through

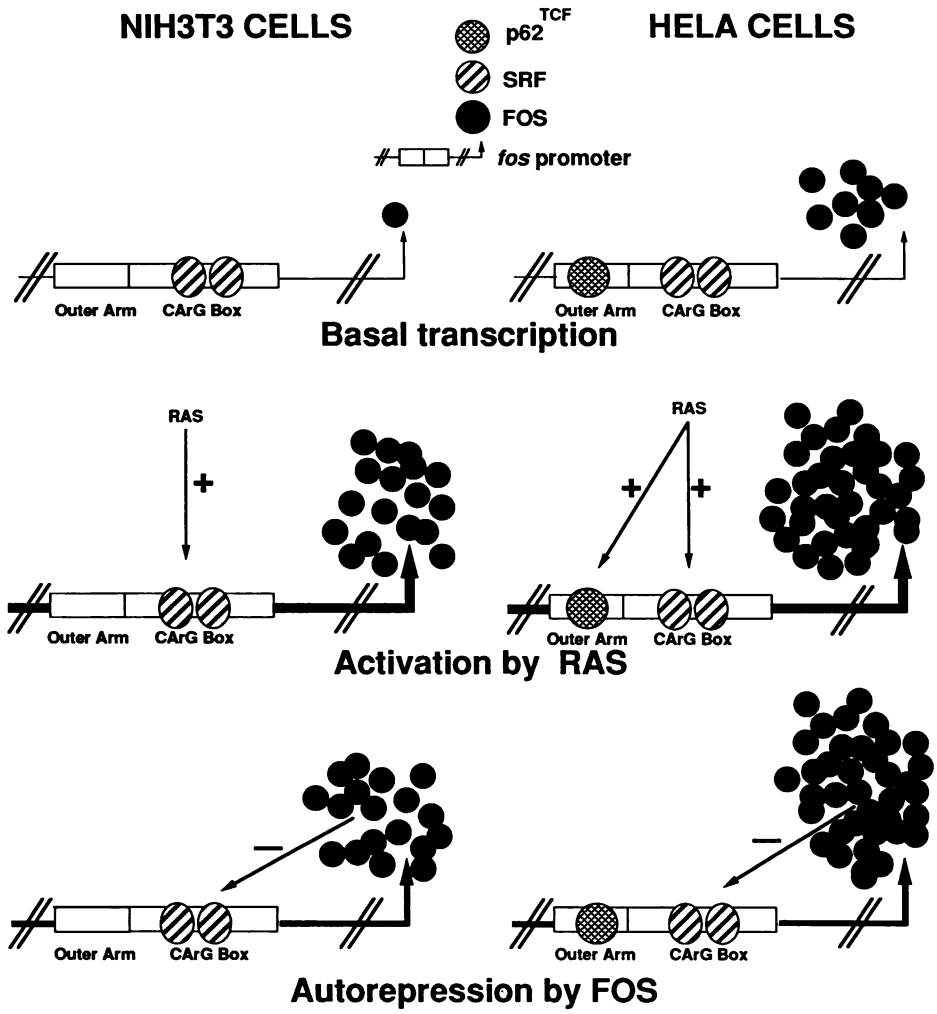

FIG. 6. Model describing cell-specific regulation of the c-*fos* promoter ORU in NIH 3T3 cells and HeLa cells. A minimal *fos* promoter encompassing the CArG box and the outer arm is depicted. Three stages (in sequential order) are considered: basal transcription, activation by *ras* expression, and autorepression by the Fos protein. Minus signs indicate negative regulation, and plus signs indicate positive regulation. The sizes of the arrows indicate rates of transcription. Mechanisms of $p62^{TCF}$ and SRF activation by *ras* are not known, but occupation of the outer arm and the CArG box does not change (14). Regulation through the outer arm plus FAP is not described in this model.

them, suggesting that they are the nuclear targets of a common signaling pathway.

The c-*fos* ORU is regulated in a cell-specific manner. In NIH 3T3 cells, the CArG box is necessary and sufficient to mediate transcription activation by *ras*. In contrast, in HeLa cells, neither of the three sequence elements alone are able to mediate transcription activation by *ras* and several other nonnuclear oncoproteins. Similarly, Velcich and Ziff (35) demonstrated that FAP does not respond to TPA in HeLa cells. The outer arm is essential for induction, but it is not sufficient. Combinations with either the CArG box or FAP respond efficiently, showing that there is cooperation between these pairs of sequences.

SRF is a transcriptional activator in NIH 3T3 cells but not in HeLa cells. We investigated the proteins that mediate the observed effects. Previous studies have shown that SRF binds to the CArG box and that $p62^{TCF}$ binds to the outer arm, but only if the SRF is present. SRF has been reported to activate transcription in vitro (21). We show that it also activates transcription through the CArG box in NIH 3T3 but not in HeLa cells. This cell-specific regulation resembles that observed for *ras*. *ras* and SRF activate through the CArG box alone in NIH 3T3 cells, suggesting that SRF is one of the mediators of the transcriptional response to *ras* expression. This activation may be mediated by casein-kinase II, a component of the mitogenic signaling pathway that phosphorylates SRF and enhances its DNA binding activity (20).

Different levels of the Fos oncoprotein contribute to the cell-specific activity of the c-*fos* promoter ORU. The level of Fos protein in the cell seems to determine the response to *ras* and SRF. With low levels of Fos, SRF can activate transcription, whereas with high levels SRF is inactive. The CArG box is sufficient to mediate a response to *ras* before Fos induction in NIH MT-FOS cells. In contrast, in induced cells, both the CArG box and the outer arm are required. Strikingly, this resembles the situation in HeLa cells, suggesting that the Fos protein itself could be a major determinant of cell-specific regulation of the c-*fos* promoter ORU. However, HeLa and NIH 3T3 cells differ in other respects, and additional factors may contribute to the effects observed.

How is the c-*fos* ORU regulated in a cell-specific manner? We propose a model to explain cell-specific regulation of the c-*fos* promoter ORU, taking into account our results and those published by others (Fig. 6). In NIH 3T3 cells in the basal state, both transcription from the *fos* promoter and Fos protein levels are low (4), and presumably expression of $p62^{TCF}$ protein is also low (which would explain the absence of an effect caused by mutating the outer arm). *ras* expression could increase SRF activity and transcription of the *fos* promoter through the CArG box. Consequently, Fos protein synthesis would repress its own promoter.

We presume that in HeLa cells, both the Fos protein and $p62^{TCF}$ are expressed at higher levels than in NIH 3T3 cells (from both the analogy with MT-FOS cells and the negative effects of mutating the outer arm). *ras* activation of $p62^{TCF}$ may help SRF overcome repression by Fos, whereas transfected SRF alone is not sufficient to activate transcription (our experiments). For *Saccharomyces cerevisiae*, an analogous regulation has been described in which MCM1, which binds to a CArG box, interacts alternatively with a coactivator or a corepressor (13, 16, 22). High $p62^{TCF}$ activity could help to maintain a high basal level of Fos despite autorepression. In this model, $p62^{TCF}$ is an important determinant of intracellular Fos levels and thus could play a critical role in transformation. It is noteworthy that the outer arm is absent in several nononcogenes that have CArG boxes in their promoters (2, 3), suggesting that this sequence has an important role.

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