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Altered Transcriptional Activity of c-*fos* Promoter Plasmids in v-*raf*-Transformed NIH 3T3 Cells

ZAHAVA SIEGFRIED AND EDWARD B. ZIFF*

Howard Hughes Medical Institute, Department of Biochemistry and Kaplan Cancer Center, New York University Medical Center, New York, New York 10016

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In cells transformed by v-*raf*, an oncogenic counterpart of the serine/threonine kinase Raf-1, regulatory elements of the c-*fos* promoter were active under conditions of cell growth or stimulation for which they were inactive in untransformed control cells. This suggests that v-*raf* transforms by deregulating transcription of early response genes.

v-*raf* is the transforming gene of the murine sarcoma virus 3611-MSV (4, 22). Its cellular counterpart, the *raf*-1 gene, encodes the Raf-1 protein, which is a cytoplasmic serine/threonine kinase (23). Mutations of *raf*-1 which truncate the amino terminus activate both the Raf-1 serine/threonine kinase function and the capacity of Raf-1 to induce cell transformation (30). Raf-1 has been implicated as a mediator of signaling between the plasma membrane and the cytoplasm. For example, a variety of mitogenic signals and membrane-associated oncogene products increase Raf-1 phosphorylation, with an associated increase in protein kinase activity (18, 19). Furthermore, microinjection of the truncated, activated Raf-1 protein induces DNA synthesis in NIH 3T3 cells (21).

Recently, Kaibuchi et al. (14) and Jamal and Ziff (12) have shown that in transient assays, constitutively activated forms of Raf-1 can induce transcription of growth factor-regulated early response genes including the c-*fos* and beta-actin genes. Multiple elements within the c-*fos* promoter are Raf-1 responsive, including the dyad symmetry element (DSE), which confers serum responsiveness on the c-*fos* promoter. In this report, we show that, unlike transient expression of v-Raf, constitutive expression of v-Raf in v-*raf*-transformed NIH 3T3 cells does not activate the DSE in the steady state. The DSE is, however, activated in v-*raf*-transformed cells by transient signals, which are insufficient to activate the DSE in a control untransformed cell. We consider the implications of these results and the effects of v-*raf* on a second element, the FAP site, in light of the mechanism of c-*fos* autoregulation. We also consider implications for the mechanism of cell transformation by v-*raf*.

Isolation of v-*raf*-transformed NIH 3T3 cells. Subconfluent dishes of NIH 3T3 cells were cotransfected with either a v-*raf* plasmid, 3611-MSV (22), and an RSVneo plasmid (2) or an RSVneo plasmid alone. The RSVneo plasmid expresses the neomycin resistance gene under the control of the Rous sarcoma virus long terminal repeat and allows selection of the cells with the antibiotic G418. After 2 weeks of culture under G418 selection, many foci were obtained on all dishes. A total of 32 foci were selected and analyzed by the polymerase chain reaction, which detects integrated v-*raf* plasmid sequences without a requirement for growth expansion of the isolated foci (15). We amplified v-*raf* sequences from DNA isolated from foci by using a pair of polymerase chain reaction primers, one complementary to the *gag* region and the other complementary to the *raf* region. Of the 32 clones analyzed, 14 yielded an amplified polymerase chain reaction fragment indicative of the integration of v-*raf* sequences (data not shown). These clones were then expanded and analyzed for the expression of v-*raf* mRNA (Fig. 1). Endogenous c-*raf* message was seen in control NIH 3T3 cells (lane 4) and NIH 3T3 cells transfected with RSVneo alone (lane 2). However, the cell line cotransfected with v-*raf* and RSVneo showed a larger transcript which corresponds to the v-*raf* mRNA (lane 1). A mixed population of pooled clones (lane 3) exhibited a lower level of the v-*raf* mRNA, as expected, since only 2 of the 14 clones with integrated v-*raf* DNA expressed detectable v-*raf* mRNA.

Altered transcriptional activity of the DSE-globin plasmid in raf-3T3 cells. To assay the effects of v-*raf* on the c-*fos* promoter, we employed two representative cell lines: raf-3T3, a cell line cotransfected with both RSVneo and v-*raf*, and neo-3T3, a control cell line transfected with only RSVneo. The raf-3T3 cells appeared to have both an altered morphology and a faster growth rate than the neo-3T3 cell line, as would be expected of a transformed cell (data not shown). We first asked how v-*raf* transformation affected the DSE, an element located at −300 bp 5′ to the cap site of the c-*fos* gene, which is necessary for the serum, growth factor, and phorbol ester induction of c-*fos* transcription (6, 8–11, 27, 31, 32). The plasmid DSE-globin (Fig. 2), which contains an oligonucleotide with the DSE sequence inserted upstream from the beta-globin promoter, was transiently transfected into raf-3T3, neo-3T3, and NIH 3T3 cells. The cells were starved for 24 h and then stimulated with serum for 4 h. Under these conditions, the DSE-globin plasmid is serum inducible in NIH 3T3 cells (28) (Fig. 3A, lane 9). Correctly initiated and spliced beta-globin transcripts were detected by the RNase protection assay by using an antisense SP6 RNA polymerase-encoded probe that is complementary to exon 1 and most of exon 2 of the beta-globin message. This assay consistently detected a basal level of constitutive expression of exon 2 sequences, which most likely resulted from aberrant initiation of transcripts which traverse the exon 2 5′ boundary. However, correctly initiated transcripts produced an exon 1-protected band defined on one side by the transcription initiation site and on the other by the 3′ end of exon 1. The DSE-globin plasmid was also serum inducible under these conditions in both raf-3T3 cells (Fig. 3A, lane 3) and neo-3T3 cells (lane 6). The DSE-globin plasmid was expressed at greatly reduced levels in both the starved control

* Corresponding author.

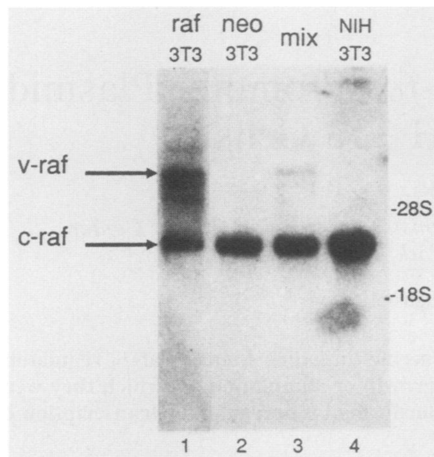

FIG. 1. Expression of v-*raf* mRNA in v-*raf*-transformed NIH 3T3 cells. Total cytoplasmic RNA was isolated from raf-3T3 cells (lane 1), neo-3T3 cells (lane 2), a mixed pool of v-*raf*-transformed cells (lane 3), or NIH 3T3 cells (lane 4). RNA (30 μg) from each sample was analyzed for the expression of v-*raf* and c-*raf* mRNA by Northern (RNA) blot analysis. The probe was a 2.9-kb *Eco*RI gel-purified fragment from a rat *raf* cDNA.

cells and the starved v-*raf*-transformed cells (lanes 1 and 4). This inactivity of the DSE when exposed to steady-state expression of v-Raf protein in v-*raf*-transformed cells is in contrast to the inducibility of the element by v-*raf* in transient assays (12, 14).

We next analyzed the effects of treatment of these transfected cells with platelet-derived growth factor (PDGF) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Figure 3B shows that the plasmid was inducible by both TPA and PDGF in the raf-3T3 cells (lanes 3 and 4), although it was not inducible in the neo-3T3 cells (lanes 7 and 8). This demonstrates that agents which are incapable of inducing the DSE-globin plasmid in control cells are effective inducers in v-*raf*-transformed cells. Taken together, these data suggest that the v-Raf protein, when expressed in the steady state, can cooperate with TPA or PDGF to induce transcription via the DSE. However, neither steady-state v-*raf* expression on its own nor treatment with either PDGF or TPA alone is sufficient to provide transcriptional activation. Induction is seen when a transient inducer (PDGF or TPA) cooperates with steady-state expression of v-*raf*.

Altered transcriptional activity of the FAP3X-globin plasmid in raf-3T3 cells. A sequence immediately 3′ to the DSE at −296 bp in the c-*fos* 5′ regulatory region resembles the consensus binding sites for the transcription factors AP-1 and CREB (13). This sequence will be referred to as the *fos* AP-1-like sequence (FAP). The FAP site has been implicated in negative regulation of the steady-state basal activity of the c-*fos* gene, although it is not necessary for down regulation of c-*fos* transcription after growth factor stimulation (16). The plasmid FAP3X-globin contains three copies of a FAP sequence oligonucleotide cloned upstream from the beta-globin surrogate gene (Fig. 2). When transfected into raf-3T3 cells, the FAP3X-globin plasmid was expressed at high levels in both starved and serum-stimulated conditions (Fig. 4A, lanes 4 to 6), while it was expressed at greatly reduced levels in the neo-3T3 cells in both starved and stimulated conditions (lanes 1 to 3). It therefore appears that there is a higher and constitutive FAP activity in the raf-3T3 cells than in the control cells.

To analyze the signals which activate the FAP3X-globin plasmid, we asked if specific agents induce this plasmid in NIH 3T3 cells. The FAP sequence shows homology to TPA-responsive elements which bind the factor AP-1 (Fig. 5) (1, 17). The FAP3X-globin plasmid was not inducible by TPA in NIH 3T3 cells under the conditions in which the DSE-globin plasmid was inducible in the raf-3T3 cells (data not shown). However, the FAP3X-globin plasmid was cyclic AMP (cAMP) inducible (Fig. 4B, lane 2). The beta-globin control plasmid that lacks the FAP sequences was not induced (lane 4), and thus induction by cAMP is through the FAP sequences and not a cryptic CRE element in the vector sequences. This induction is in agreement with previous reports of cAMP inducibility of plasmids containing the FAP element (3, 7). These data suggest that the higher expression of the FAP3X-globin plasmid in raf-3T3 cells may reflect activation of a cAMP-dependent pathway in these cells. The FAP3X-globin plasmid is also stimulated by cAMP in PC12 cells but not in a mutant PC12 cell line that lacks protein kinase A (A. Velcich and E. Ziff, Mol. Cell. Biol., in press).

We have attempted to determine the protein target(s) of v-Raf in our transformed cells. By a gel mobility shift assay, we could detect specific binding of factors to the DSE and FAP oligonucleotides. However, we could not detect any differences in this binding in nuclear extracts from v-*raf*-transformed cells compared with those from normal cells (data not shown). Possibly v-Raf modifies factors that bind to these sites that cannot be detected by the mobility shift assay. One such modification could be phosphorylation of the factor itself or an intermediate factor that induces its transcriptional activity, as has been shown for CREB (35).

Deregulation of transcription as a basis for the oncogenic potential of v-*raf*. In this paper, we report altered transcriptional activity in v-*raf*-transformed NIH 3T3 cells of two plasmids bearing elements from the c-*fos* promoter. In a v-*raf*-transformed cell, the plasmid DSE-globin is responsive to two agents, TPA and PDGF, which are incapable of inducing activity in control cells. Thus, constitutive expression of an activated form of the Raf-1 kinase can alter the transcription induction requirements of a regulated c-*fos* promoter element. The FAP3X-globin plasmid is constitu-

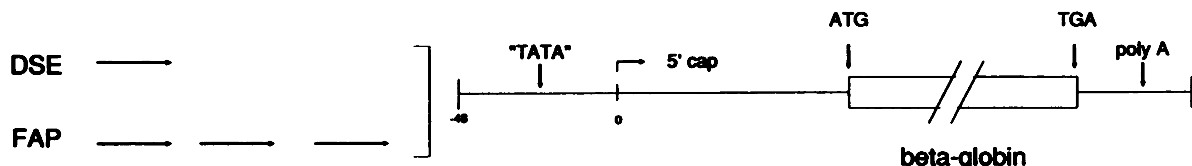

FIG. 2. Structure of beta-globin constructs. A genomic clone of human beta-globin containing 48 nucleotides 5′ of the cap site and approximately 500 nucleotides 3′ of the poly(A) site was cloned into the pUC13 vector. DSE-globin contains one copy of a synthetic DSE oligonucleotide inserted in the polylinker of pUC13. FAP3X-globin contains three tandem copies of a synthetic FAP oligonucleotide inserted in the polylinker; arrows indicate orientation.

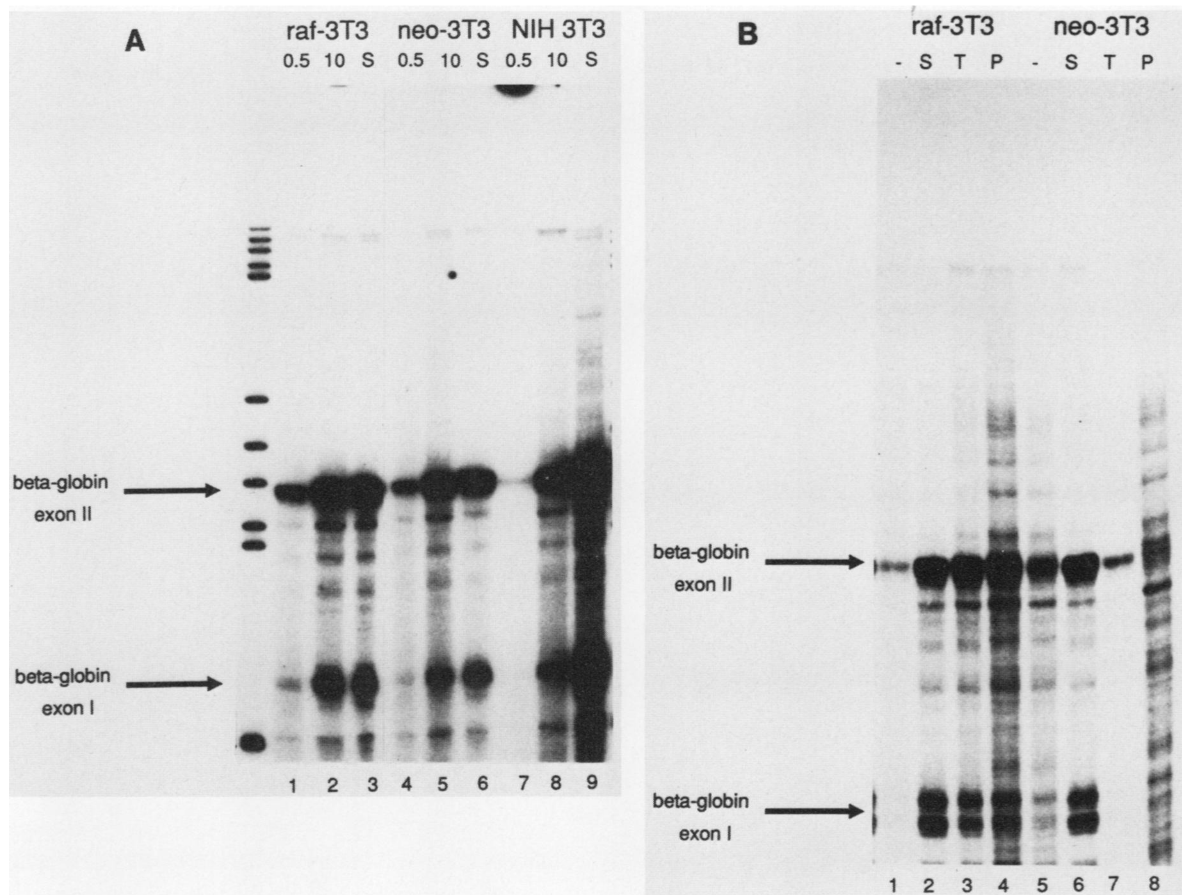

FIG. 3. Transient transfection of DSE-globin plasmid in v-*raf*-transformed cells. (A) DSE-globin plasmid (20 μg) was transiently transfected into either raf-3T3 cells (lanes 1 to 3), neo-3T3 cells (lanes 4 to 6), or NIH 3T3 cells (lanes 7 to 9). Cytoplasmic RNA was isolated after cells were either serum starved in Dulbecco modified Eagle medium plus 0.5% calf serum for 24 h (lanes 1, 4, and 7), maintained in normal growth Dulbecco modified Eagle medium plus 10% calf serum (lanes 2, 5, and 8), or serum starved and then stimulated by the addition of 15% calf serum for 4 h (lanes 3, 6, and 9). (B) DSE-globin plasmid (20 μg) was transiently transfected into either raf-3T3 cells (lanes 1 to 4) or neo-3T3 cells (lanes 5 to 8). Cells were serum starved in Dulbecco modified Eagle medium plus 0.5% calf serum for 24 h, and cytoplasmic RNA was isolated either before stimulation (lanes 1 and 5) or 4 h after stimulation by either 15% calf serum (lanes 2 and 6), 260 ng of TPA per ml (lanes 3 and 7), or 10 ng of recombinant PDGF per ml (lanes 4 and 8). In both panels, human beta-globin mRNA was quantitated by the RNase protection assay. Beta-globin exon I, a protected 142-bp human beta-globin mRNA fragment; beta-globin exon II, a protected 203-bp human beta-globin mRNA fragment.

tively active in v-*raf*-transformed cells but inactive in control cells, both in the presence and the absence of a serum stimulus. This suggests that v-*raf* can provide a signal which activates the FAP element and that a comparable signal is not provided by serum. The fact that cAMP will activate the FAP3X-globin plasmid raises the possibility that v-*raf* activates the FAP element via a cAMP-regulated mechanism.

Several lines of evidence implicate Raf-1 in the transmission of signals from the plasma membrane to the nucleus. Raf-1 is phosphorylated in response to a variety of mitogenic signals and membrane-bound oncogene products (19). Although Raf-1 is a cytoplasmic kinase, Raf-1 has been reported to undergo rapid redistribution to the nucleus and perinuclear space in response to certain mitogenic factors (23), where it could modify nuclear factors in response to transmembrane signals. Because microinjection of antibodies to *ras* protein blocks transformation by v-*src*, but not by *mos* or *raf*, *raf* may act downstream of *ras* in the signal transduction pathway (29). Oncogene products, such as v-*src*, polyomavirus middle-T antigen, and *ras*, which increase Raf-1 phosphorylation and associated kinase activity also activate the PEA1 motif (murine AP-1 motif) (33). Indeed, it has recently been shown that the expression of v-*raf* can activate the PEA1 motif and that the ATP-binding activity of v-*raf* is required for transactivation of the PEA1 motif (34), which is consistent with its action as a kinase.

Recently, Kaibuchi et al. (14) and Jamal and Ziff (12) have shown that transient expression of activated forms of *raf*-1 or v-*raf* can induce the transcriptional activity of promoters reliant upon the DSE. In contrast, in our experiments steady-state expression of v-*raf* in the transformed 3T3 cells did not lead to activity of the DSE-globin plasmid. Potentially, this difference reflects two features of c-*fos* regulation. The c-*fos* promoter is generally responsive to transient stimuli such as those provided by serum treatment of quiescent cells (11). Also, it is inactive in the steady state, for example when growing cells are continuously exposed to serum (5). The latter inactivity most likely reflects the ability of c-Fos to regulate its own expression by a mechanism which acts through the DSE (24–26). Our experiments analyze the activity of the DSE-globin plasmid when introduced into a cell which expressed v-Raf in the steady state. The

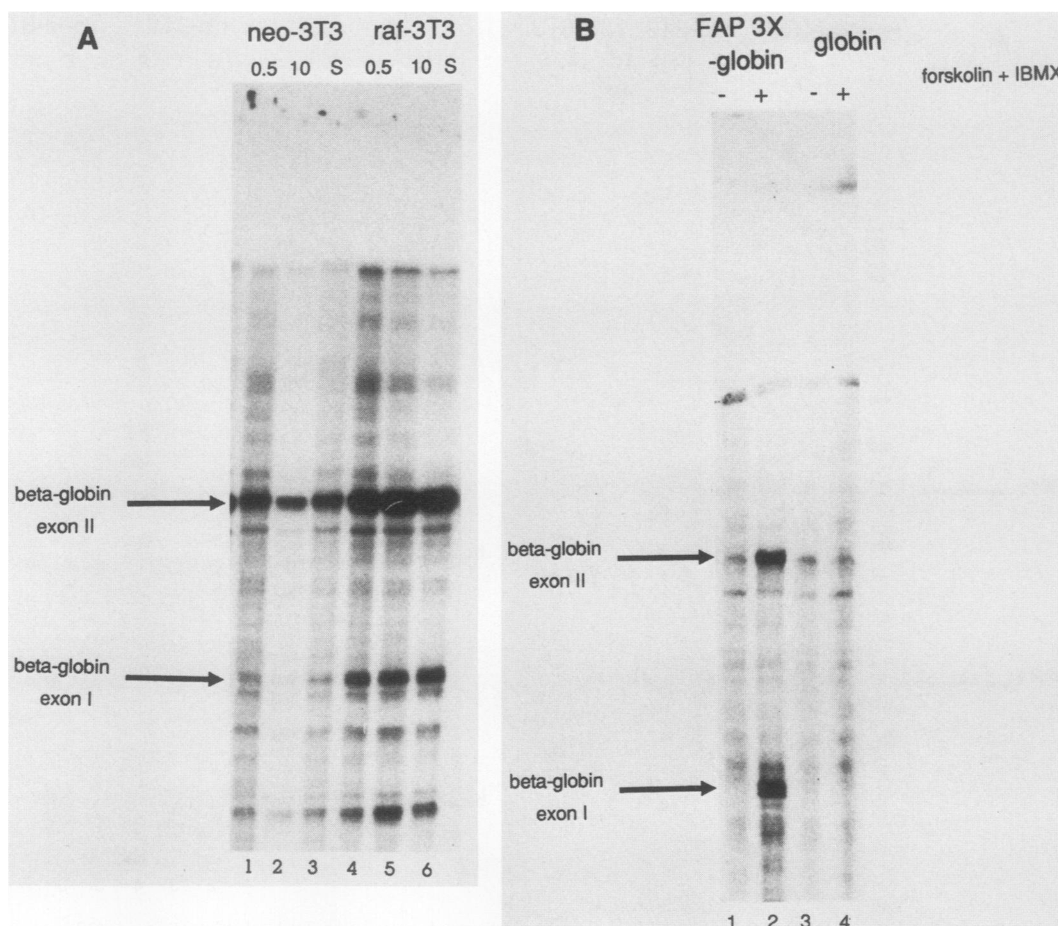

FIG. 4. Transient transfection of the FAP3X-globin plasmid in v-*raf*-transformed cells and NIH 3T3 cells. (A) FAP3X-globin plasmid (20 μg) was transiently transfected into either neo-3T3 cells (lanes 1 to 3) or raf-3T3 cells (lanes 4 to 6). Cytoplasmic RNA was isolated after cells were either starved in 0.5% calf serum for 24 h (lanes 1 and 4), grown in normal 10% calf serum for 24 h (lanes 2 and 5), or starved for 24 h and then stimulated by the addition of 15% calf serum for 4 h (lanes 3 and 6). (B) NIH 3T3 cells were transiently transfected with either FAP3X-globin (lanes 1 and 2) or globin (lanes 3 and 4). Cells were serum starved for 24 h, and cytoplasmic RNA was isolated either before (lanes 1 and 3) or 4 h after (lanes 2 and 4) stimulation by 20 μM forskolin and 0.5 mM isobutylmethylxanthine (IBMX). In both panels, human beta-globin mRNA was quantitated by the RNase protection assay. Beta-globin exon I, a protected 142-bp human beta-globin mRNA fragment; beta-globin exon II, a protected 203-bp human beta-globin mRNA fragment.

fact that the DSE-globin plasmid is inactive under these conditions suggests that it associates with factors, including perhaps c-Fos, that generate a transcriptionally inactive complex. Because the plasmid in these transfected cells may be induced by TPA and PDGF, agents which are unable to induce in control untransformed cells, v-Raf expression does alter the transcriptional potential of the plasmid. The molecular basis for this ability of TPA or PDGF to induce the DSE-globin plasmid in v-*raf*-transformed cells is not known.

A second plasmid, FAP3X-globin, does show a change in

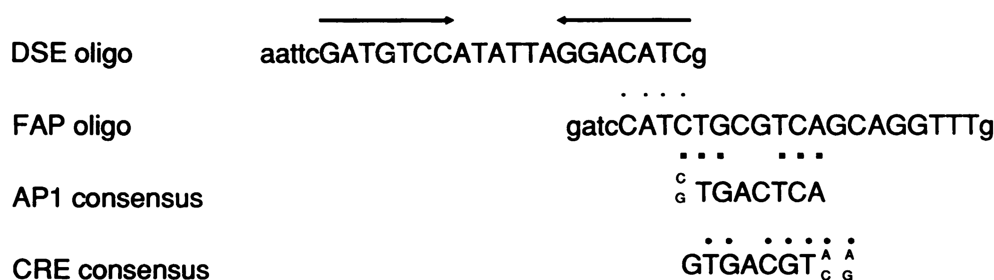

FIG. 5. Sequence comparisons of synthetic oligonucleotides and AP-1 and CRE consensus sequences. The sequences of the DSE and FAP synthetic oligonucleotides are shown. Lowercase letters are nucleotides that formed restriction enzyme sites and that are not present in the c-*fos* sequence. Squares show where the FAP and DSE oligonucleotides overlap in sequence. Aligned underneath the FAP sequence are consensus sequences for AP-1 and CRE (13). Either squares or circles above them show where they share homology with the FAP sequence.

steady-state activity in the v-*raf*-transformed cells, whereas it is highly active in the presence or absence of serum. This element, which is present in the c-*fos* promoter in a single copy adjacent to the DSE, is not thought to be required for c-*fos* autoregulation. Thus, it is not surprising that in contrast to the DSE, it is not subject to steady-state repression. Our results also show that FAP3X-globin may be activated by a cAMP-dependent pathway, although the relevance of cAMP regulation to its activity in v-*raf*-transformed cells remains to be established. The c-*fos* promoter contains a single copy of both the DSE and FAP sites (31). A single copy of the FAP element is a less potent activator of transcription than the threefold tandem repeat analyzed here (A. Velcich and E. Ziff, submitted). The contribution of a single FAP element to the activity of the c-*fos* promoter in a v-*raf*-transformed cell remains to be determined. Proteins binding to the FAP and DSE elements may interact physically or mechanistically. These results suggest that the regulatory properties of factors binding to both elements are altered by v-*raf*. The effects of v-*raf* described here raise the possibility that v-*raf* transforms by deregulating an early response gene(s) which controls cell cycle progression, as suggested for the transcription factor AP-1 (20, 33).

We recently reported the increased ability of TPA to activate the plasmid DSE-globin in HeLa cells versus NIH 3T3 cells (28), similar to that found here for v-*raf*-transformed cells versus control cells. We suggested that this deregulation may be due to some aspect of the transformed state of HeLa cells which constitutively provides a component of the signals which are required for DSE activation. In this work, we demonstrate similar effects by a defined oncogenic modification of NIH 3T3 cells, expression of the v-Raf protein. Perhaps proto-oncogenes which participate in the growth factor-induced signal transduction pathway, such as v-*raf*, converge on a single pathway which controls a small number of genes, such as c-*fos*, which in turn can affect the processes of growth and differentiation. Further studies of the targets of such proto-oncogenes, including Raf-1, should help to elucidate this process.

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