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Oncogene activation of HIV-LTR-driven expression via the NF-κB binding sites

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ABSTRACT

The Raf-1 proto-oncogene product is a highly regulated serine/threonine kinase that functions in signal transduction downstream from growth factor receptors and upstream from nuclear proto-oncogene products. Using a transient cotransfection assay we have found that activated Raf-1 activates expression from the HIV-LTR. Analysis of a series of 5′ deletion and point mutations revealed the NF-κB motifs as the Raf-responsive element in the HIV-LTR. Moreover, Raf-BXB activated expression from heterologous promoters driven by the HIV NF-κB binding sites. In addition to Raf, we show that v-Src, v-H-Ras and v-Mos activate HIV-LTR expression through the NF-κB binding sites and v-H-Ras-induced HIV-LTR expression is mediated by Raf-1. These findings may have implications for the involvement of the cellular homologues of these oncogenes in the switch from latent to productive infection by HIV in response to T-cell activation.

INTRODUCTION

The Raf-1 proto-oncogene product plays a key role in transducing signals from growth factor receptors at the cell surface to the nucleus. Stimulation of growth factor receptors on quiescent cells results in Raf-1 hyperphosphorylation and kinase activation (1,2). *c-raf-1* can be oncogenically activated by deletions, insertions or point mutations in the regulatory domain, conserved regions 1 or 2 (3,4,5, J.T.B., G.H. and U.R.R., unpublished data). In addition to transforming NIH 3T3 cells, activated Raf-1 activates expression from AP-1, Ets, and SRF binding sites (6–10).

Several lines of evidence indicate that v-Src functions in the activation of Raf-1. First, Raf-1 is hyperphosphorylated and displays constitutively activated kinase activity in cells transformed by v-Src (1,11). Second, v-Src induces Raf hyperphosphorylation and activation when the two proteins are over-expressed in Sf-9 cells (12). Third, a Raf-1 dominant-negative mutant efficiently blocked v-Src induced expression from the Egr-I promoter in transient assays (13) and v-Src-induced Jun phosphorylation and activation (14). In addition, Lck, another member of the Src family tyrosine kinases, has been implicated in Raf-1 phosphorylation and activation in response to CD4 receptor crosslinking and IL-2 stimulation in T cells (16–20). Similarly, Ras is involved in the activation Raf-1 following stimulation of growth factor receptors. We have shown that a Ras dominant negative mutant, Ras-N-17, blocks Raf-1 activation in response to serum, TPA and NGF treatment of cells (21). In addition, a dominant negative Raf-1 mutant blocks Ras induced transformation (22) and transactivation of AP-1/Ets-dependent gene expression (10). Although Ras clearly functions in the activation of Raf-1, experiments by Reed *et al.*, (11) demonstrated the involvement of a growth factor signal in addition to activated Ras in Raf-1 activation.

Treatment of cells harboring latent HIV provirus with cytokines or phorbol esters results in the activation of the transcription factor, NF-κB, an increase in transcription from the HIV-LTR and a switch to productive infection (23,24). Since Raf-1 activation by cytokines and phorbol esters has been well documented (reviewed in 2) and Raf-1 is a necessary component of the mitogenic signalling cascade (22), acting at least in part by activating expression of genes driven by AP-1 and Ets binding motifs (6, 7, 10), we examined the ability of activated Raf-1 to transactivate expression from the HIV-LTR and found that the Raf-responsive element corresponds to the NF-κB binding sites. In addition, we show that expression from the NF-κB binding sites in the HIV-LTR is induced by the v-Src, v-H-Ras and v-Mos oncogene products. As NF-κB binding proteins belong to the Rel proto-oncogene family (25–29), our findings support the notion that activated Raf-1 promotes a program of cell growth by activating nuclear proto-oncogene products.

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EXPERIMENTAL PROCEDURES

Cells and transfections

Monolayer cultures of NIH 3T3 cells were grown in Dulbecco's Modified Eagle Medium containing 10% fetal calf serum. Jurkat cells were grown in RPMI Medium 1640 containing 10% fetal calf serum.

NIH 3T3 transfections were performed by the calcium phosphate precipitation method (30). Cells (10^6) were seeded one day prior to transfection. The cells were transfected with a reporter construct, an HIV Tat expression vector, vectors expressing the various oncogenes and salmon sperm DNA such that the final concentration of DNA was 20 μg/transfection. After an overnight incubation the cells were washed and then incubated in fresh media containing 0.3% fetal calf serum. Jurkat cells (10^7) were transfected using DEAE-dextran (37). Briefly, (10^7) cells were pelleted and resuspended in 1 ml of TS containing 1 μg L3B-CAT 0.5 μg HIV tat expression vector, 10 μg Raf-BXB or kRSPA expression vector and 0.5 mg DEAE-dextran. After a 15 min. incubation at room temperature 10 ml of RPMI medium containing 5% fetal calf serum and 100 μM chloroquine was added and the cells were incubated for 30 min. at 37°C. The cells were subsequently washed with RPMI medium containing 5% fetal calf serum, resuspended in medium containing 0.3% fetal calf serum and incubated at 37°C for 48 hours. Total cell extracts were prepared 48 hours after transfection and CAT enzyme levels were assayed by the diffusion-based CAT assay as described by the manufacturer (NEN). Transfections were performed at least three times with at least two different plasmid DNA preparations.

Plasmids and site-directed mutagenesis

Plasmids expressing Raf-1 and various Raf mutants from the RSV-LTR and the v-H-Ras and v-Mos expression vectors have been previously described (10). RSV-Raf-C4B was generated by ligating an oligo encoding the C-terminal 17 amino acids of B-Raf to the large *Sal*-I to *Xba*-I fragment from RSV-Raf-1 (10). L3CATt, L3BCAT, L3CAT-91 and L3CAT-33 have been previously described (31). v-Src was expressed from the Moloney murine leukemia virus long terminal repeat in a vector termed pMv-Src (32). Point mutations in the GM-CSFRE and the NF-κB binding sites were generated by PCR-directed mutagenesis using standard techniques (33). The nature of the point mutations was confirmed by nucleotide sequence analysis.

RESULTS

To determine if activated Raf-1 was capable of transactivating expression from the HIV-LTR, NIH 3T3 cells were cotransfected with an HIV-LTR-CAT reporter construct and expression vectors containing the *c-raf-1* gene, two mutants of *c-raf-1* that increase Raf-1 transformation functions, Raf-BXB and Raf-pm17, or a mutant that functions as a dominant inhibitor with regard to transformation, Raf-301 (Fig. 1A). Overexpression of wild-type Raf-1 activated expression 2-fold from the HIV-LTR. Mutations in Raf-1 that activated the transforming activity increased the transactivation potential of Raf-1 and a mutant that abolished the transforming activity did not activate transcription from the HIV-LTR (Fig. 1B).

The HIV-LTR contains binding motifs for several transcription factors (Fig. 2A). To determine the sequences in the HIV-LTR necessary for Raf activation of transcription we analyzed a series

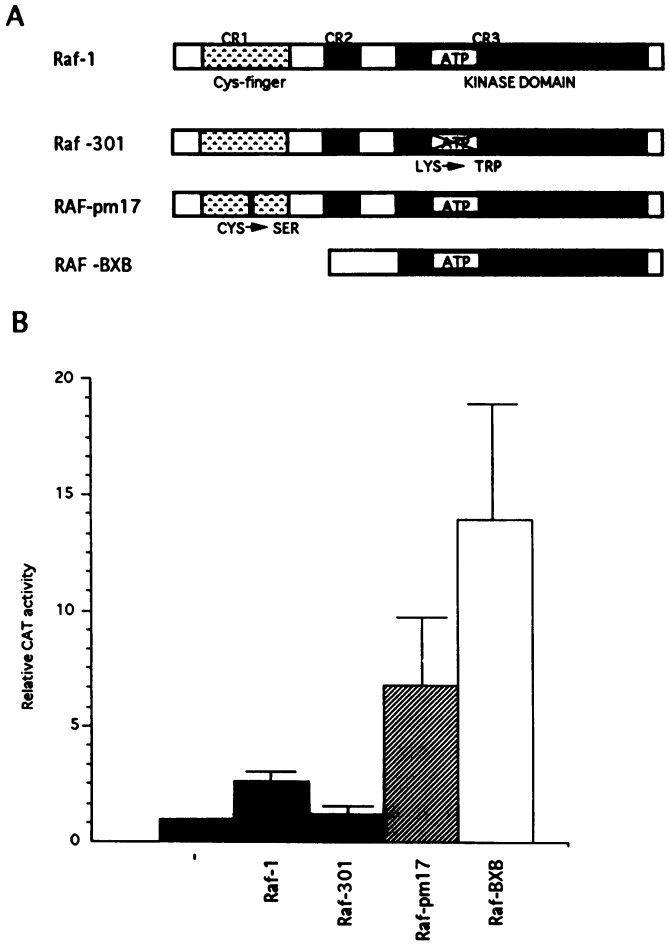

Figure 1. Raf-1 transactivates expression from the HIV-LTR. **A.** A schematic representation of Raf-1 expression constructs tested for transactivation is shown. The positions of the Raf family conserved regions, CR1, CR2, and CR3, are indicated. **B.** The indicated RSV-Raf-1 expression vectors were transfected into NIH 3T3 cells with L3-CATt and a Tat expression vector, pRS-Htat. One unit of relative CAT activity is equivalent to the level of CAT activity observed in the L3-CATt, pRS-Htat cotransfection. The standard deviations of the mean values are indicated by error bars.

of 5′ deletions in the HIV-LTR. A reporter construct containing a deletion to nt −120, L3B-CAT, was fully responsive to Raf-BXB activation indicating that the AP-1, NFAT-1 and USF-1 binding sites are not necessary for Raf activation of transcription (Fig. 2). This is in contrast to the situation in the oncogene-responsive element in the polyomavirus enhancer where the AP-1 and Ets binding sites confer Raf-1 responsiveness (6,7,10). The increase in basal levels of expression in L3B-CAT versus L3-CATt presumably reflects the presence of the negative regulatory element in the latter (34). Deletion to nt −91, L3-CAT-91, removed the upstream NF-κB site and significantly reduced Raf responsiveness. Deletion to nt −33, L3-CAT-33, abolished induction by Raf-BXB (Fig. 2). These deletion mutants revealed the importance of two regions for transactivation by activated Raf-1. The first region lies between nt −120 and nt −91 and the second region lies between nt −91 and nt −33. These two Raf-responsive regions share a common enhancer element, the NF-κB binding site. In addition to the NF-κB binding site, the upstream Raf-responsive region contains a GM-

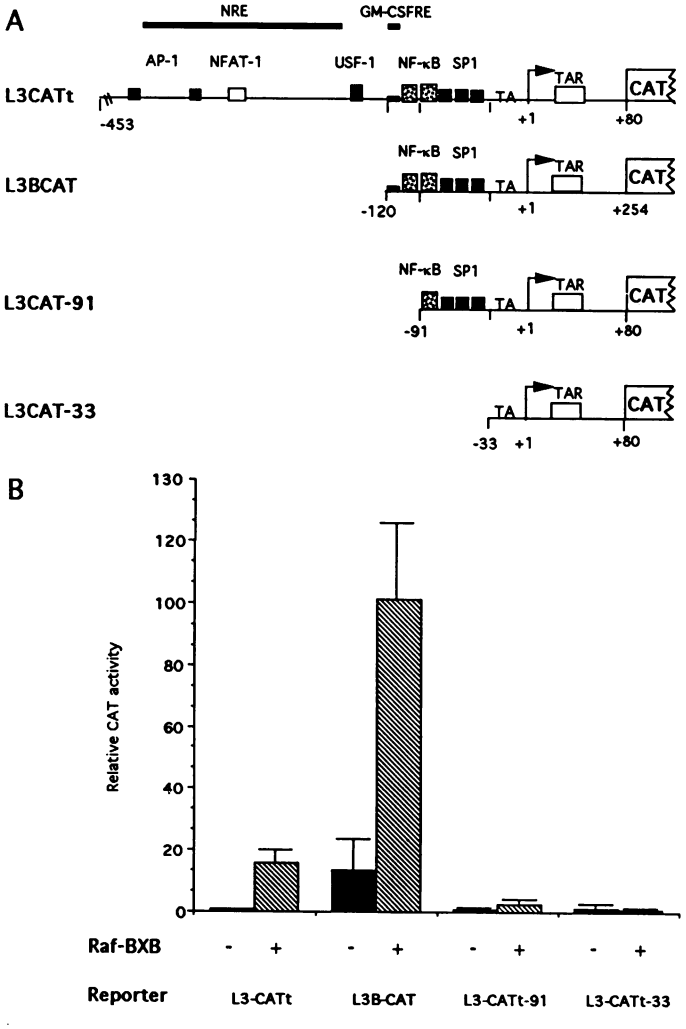

Figure 2. Identification of a Raf-responsive element in the HIV-LTR. **A.** Schematic representation of the HIV U3-R regions as they appear in L3CATt. The location of the negative regulatory element (NRE), a GM-CSF responsive element, the TATA box (TA), the TAR region and binding sites for AP-1, NFAT-1, USF-1, NF-κB and SP-1 are indicated. The arrow at +1 indicates the transcriptional start site. Deletions in L3-CATt (L3BCAT, L3CAT-91 and L3CAT-33) are shown (31). **B.** NIH 3T3 cells were cotransfected with the indicated reporters and pRS-Htat with or without Raf-BXB. The standard deviations of the mean values are indicated by error bars.

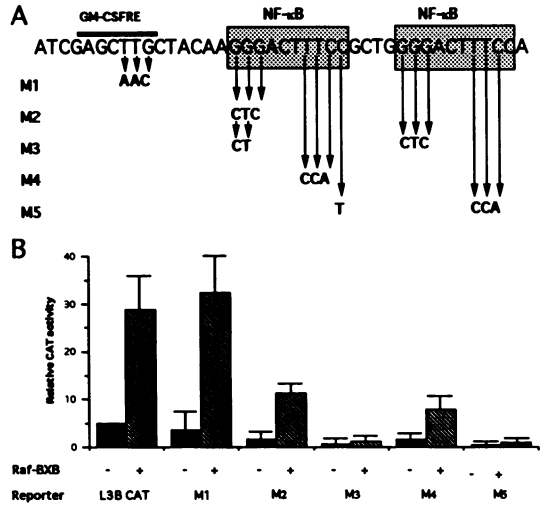

Figure 3. The NF-κB binding site is Raf-responsive. **A.** A schematic representation of the Raf-responsive element in the HIV-LTR. The sequence of the Raf-responsive element in the HIV-LTR from −120 to −80 relative to the start site of transcription is shown. The GM-CSFRE and NF-κB binding sites are indicated. M1, M2, M3, M4 and M5 are L3B-CAT derivatives which contain three consecutive point mutations in the GM-CSFRE, the upstream NF-κB binding site or mutations in both the upstream and downstream NF-κB binding sites, as indicated. **B.** Reporter constructs carrying the indicated point mutations in an L3B-CAT background and pRS-Htat were cotransfected into NIH 3T3 cells with or without Raf-BXB, and CAT enzyme levels were determined. The standard deviations of the mean values are indicated by error bars.

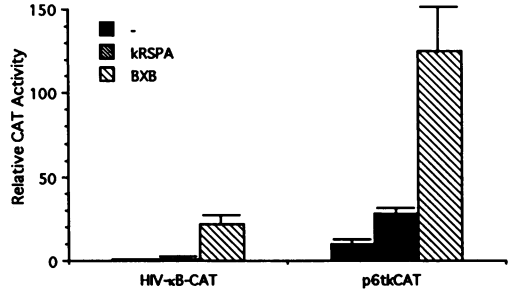

Figure 4. Activated Raf transactivates heterologous promoters driven by the HIV NF-κB binding sites. NIH 3T3 cells were cotransfected with 5 μg of p6tkCAT or HIV-κB-CAT and 5 μg of Raf-BXB or kRSPA as indicated. kRSPA, the empty control vector, contains the RSV-LTR and SV40 polyadenylation signal inserted into bluescript KS (44). The columns show the relative CAT activity (relative to HIV-kB-CAT alone) and represent the mean of three independent experiments. Error bars indicate standard errors of the mean.

CSF responsive element (GM-CSFRE) (35) and the downstream Raf-responsive region contains three SP-1 binding sites.

To identify which element is important for Raf-1 activation of transcription we generated point mutations in these transcriptional elements in an L3B-CAT background (Fig. 3A). These mutants were tested for Raf activation using Raf-BXB in NIH 3T3 cells. Three consecutive point mutations in the GM-CSFRE, M1, had no effect on transactivation by Raf-BXB. Conversely, three consecutive point mutations in the 5′ half site, M2, or 3′ half site, M4, of the upstream NF-κB binding site, reduced Raf induced CAT expression by about 50% (Fig. 3B). The increased Raf responsiveness of M2 versus L3CAT-91 likely reflects weak binding of the Raf target transcription factor to the M2 mutant. To determine the contribution of the downstream NF-κB binding site in Raf transactivation we coupled upstream NF-κB binding site mutants to similar mutations in the downstream NF-κB site and analyzed their effect on Raf-BXB transactivation. The double mutants, M3 and M5, abolished transactivation by Raf-BXB (Fig. 3B) indicating that both NF-κB binding sites in the HIV-LTR function as Raf-responsive elements.

To determine if the context of the NF-κB binding sites within the HIV-LTR was important, the Raf-responsiveness of the NF-κB sites linked to heterologous promoters was evaluated. The reporters, p6tkCAT (36) and HIV-κB-CAT (37), contain CAT genes driven by the HIV NF-κB binding sites inserted upstream of the TK promoter or the c-fos minimal promoter, respectively. Cotransfection of p6tkCAT or HIV-κB-CAT with Raf-BXB

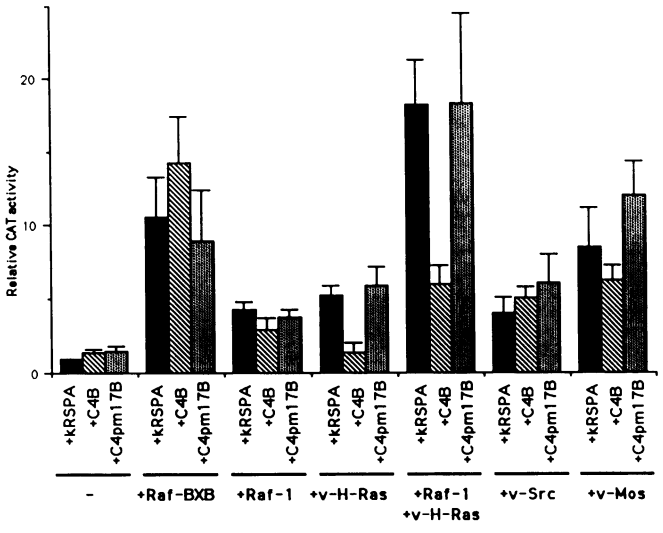

Figure 5. Oncogene activation of HIV-LTR-driven expression. NIH 3T3 cells were cotransfected with 1 μg of L3B-CAT and 0.5 μg of pRS-Htat and 5 μg each of the indicated expression vectors. v-H-Ras expresses activated Ras from the SV40 early promoter. v-Mos was expressed from the Moloney murine leukemia virus long terminal repeat in a vector termed pM-1 and v-Src was expressed from the Moloney murine leukemia virus long terminal repeat in a vector termed pMv-Src (32). The Raf expression vectors express Raf-1, Raf-BXB, Raf-C4B or Raf-C4pm17B from the RSV-LTR. One unit of relative CAT activity is equivalent to the level of CAT activity observed in the L3B-CAT, pRS-Htat cotransfection. The columns represent the mean of three independent experiments. Error bars indicate standard errors of the mean.

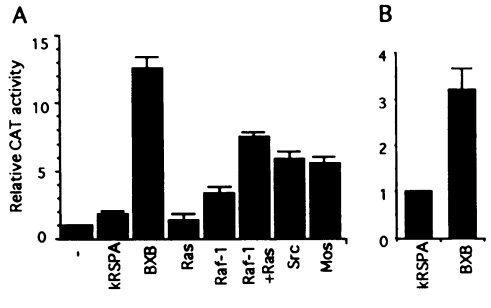

Figure 6. Tat is not required for oncogene-induced HIV-LTR-driven expression, and transactivation by activated Raf in Jurkat T cells. **A.** NIH 3T3 cells were transfected with 5 μg of L3B-CAT and 5 μg each of the indicated expression vectors. **B.** Jurkat cells were transfected with 1mg of L3B-CAT, 0.5 μg of pRS-Htat and 5mg of either RSV-BXB or kRSPA as indicated, using DEAE dextran. The columns show the relative CAT activity (fold induction) and represent the mean of three independent experiments. Error bars indicate standard errors of the mean.

resulted in an increase in CAT activity relative to the vector control, kRSPA (Fig. 4). Since RSV-BXB did not transactivate similar control reporters that did not carry the HIV NF-κB binding sites (data not shown), these results indicate that activated Raf modulates expression from the HIV NF-κB binding sites irrespective of their promotor context.

v-Src, v-H-Ras and v-Mos activate expression from the AP-1/Ets binding sites in the polyomavirus enhancer (38). Since v-Src and v-H-Ras are involved in Raf-1 activation, we next determined if these oncogene products could induce expression from the NF-κB binding sites in the HIV-LTR. NIH 3T3 cells were cotransfected with the L3B-CAT reporter and expression vectors encoding these oncogenes. v-Src, v-H-Ras and v-Mos efficiently transactivated CAT expression (Fig. 5). Since reporters carrying point mutations in both NF-κB binding sites were not responsive to v-Src, v-H-Ras, and v-Mos expression, these results indicate that the NF-κB binding sites are the target for transactivation by these oncogene products (data not shown).

The dependence on Raf-1 for v-H-Ras-induced expression through NF-κB binding sites was investigated in two ways. Cotransfection of vectors expressing Raf-1 and v-H-Ras lead to a synergistic increase in HIV-LTR-driven CAT expression. Furthermore, cotransfection of a dominant-negative Raf-1 mutant (RSV-Raf-C4B) blocked v-H-Ras- but not v-Src-, and v-Mos-induced transactivation of L3B-CAT expression (Fig. 5). A single amino acid substitution in the cysteine-rich region of Raf-C4B, Raf-C4pm17B, inactivated the inhibitory effect of Raf-C4B on Ras-induced HIV-LTR-driven expression. Since Raf-C4B functions by blocking the activation of Raf-1 (10), these results indicate that Raf-1 is required for Ras-induced activation of expression through NF-κB binding sites.

The Tat gene product is a strong activator of HIV-LTR-driven expression. To determine if the above oncogene products require Tat for activation of expression from the NF-κB binding sites in the HIV-LTR we cotransfected cells with L3B-CAT and vectors expressing the various oncogene products in the absence of Tat. Although the levels of expression are reduced in the absence of Tat, each of the oncogene products, with the exception of v-H-Ras, which was the weakest transactivator in the presence of Tat, efficiently induced CAT expression in 3T3 cells (Fig. 6A).

Since activation of Jurkat cells induces expression from the HIV-LTR, we next examined the effect of activated Raf on HIV-LTR-driven expression. Jurkat cells were cotransfected with L3B-CAT and either RSV-BXB or the vector control, kRSPA, and CAT expression was monitored. Activated Raf transactivated expression from the L3B-CAT reporter 3-fold, indicating that Raf can activate HIV-LTR expression in a cell line that is susceptible to infection by HIV.

DISCUSSION

In this report we have shown that Raf-1 activates HIV-LTR-driven expression in a Tat-independent fashion. Mutations in Raf-1 that activate its transforming functions and increase its ability to activate expression from AP-1/Ets-driven promoters also increased its activity on HIV-LTR-driven expression. Raf activation of HIV-LTR expression was dependent on the NF-κB binding sites as deletions or point mutations that disrupt NF-κB binding *in vitro* (37) blocked transactivation by Raf-BXB. Moreover, the HIV NF-κB binding sites were sufficient for Raf transactivation as they conferred Raf responsiveness on hererologous promoters.

v-Src, v-H-Ras and v-Mos also activated HIV-LTR-driven expression through the NF-κB binding sites. Transactivation by v-H-Ras was blocked by a dominant-negative Raf mutant, Raf-C4B, and v-H-Ras synergized with Raf-1 for transactivation. The Raf-C4B mutant was inactivated by a point mutation in the cysteine-rich region suggesting that this domain is necessary for the dominant-negative effect. We have previously shown that v-H-Ras mediates activation of Raf-1 kinase through interactions with this motif and that activation of Raf-1 resulted in increased expression of AP-1/Ets-driven reporters (10). v-Src and v-Mos transactivation was not sensitive to repression by RSV-Raf-C4B.

This was expected for v-Mos which may be acting either downstream or in a different pathway than Raf-1. However, the findings with v-Src were somewhat surprising in that we have previously determined the positioning of these oncogene products in a linear pathway with the order Src−Ras−Raf/Mos. Thus, v-Src may activate more than one pathway that result in increased transcription through NF-κB binding sites, only one of which involves Raf-1. Alternatively, the mechanism by which v-Src activates Raf-1 may be insensitive to the inhibitory effects of the Raf-C4B mutant. Whether Src-induced transcription through NF-κB binding sites requires a Raf-1 signalling pathway or other kinases requires further investigation.

Following activation Raf-1 initiates a kinase cascade, the outcome of which is an increase in transcription through AP-1 binding sites and cell transformation. Specifically, activated Raf-1 kinase phosphorylates and activates a 50-kD protein, Map kinase-kinase, which in turn phosphorylates and activates Map kinase (40,41). Map kinase has been shown to phosphorylate Jun at two activating serine residues, serines 63 and 73 (42), and the Raf dominant-negative mutant blocks phosphorylation of Jun in response to v-H-Ras (14). As Raf-1 is required for expression through SRE's (13) and Ets binding sites (10), and activates expression through NF-κB binding sites, it remains to be seen whether these additional effects are direct, mediated by Map kinase or a different kinase cascade.

The M3 point mutations in the HIV-LTR that abolished oncogene responsiveness have previously been shown to block PMA and PHA-induced expression from the HIV-LTR. Moreover, these mutations prevent NF-κB (p50, p65) and c-Rel binding *in vitro* (39, 43). Several lines of evidence suggest that Raf does not function by inducing the phosphorylation and activation of the p50, p49 (p50B) and p65 NF-κB binding proteins or by inactivating I-κB (MAD-3). First, we fail to observe transcriptional synergy in cotransfection experiments between activated Raf and the p50, p49 (p50B) or p65 gene products (data not shown). Second, point mutations in the 3′, non-conserved, NF-κB half site, M5, did not disrupt Rel or p50 binding *in vitro* (data not shown), but abolished Raf-BXB responsiveness (Fig. 5). Third, activated Raf-1 and activated Map kinase failed to phosphorylate I-kB, p50 or p50B *in vitro* (data not shown). Fourth, nuclear extracts from Raf-transformed NIH 3T3 or Jurkat cells did not display altered NF-κB binding activity in gel shift assays relative to extracts from control cells. Thus, although Raf-induced transactivation of the HIV-LTR requires the NF-κB binding sites, it appears that it may not function through the classical NF-κB binding proteins.

Regardless of the precise mechanism by which v-Src, v-Ras, v-Mos and activated Raf function, it is clear that they are important activators of HIV expression. As Src family members, Ras and Raf-1, are activated by many of the same agents which induce transcription from the HIV-LTR, these proto-oncogene products may play an important role in the switch from latent to productive infection by HIV.

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