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Expression of *raf* Oncogenes Activates the PEA1 Transcription Factor Motif

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PEA1 (AP1) motif transcription enhancer activity was stimulated by v-*raf* and more efficiently by activated c-*raf*-1 or A-*raf* than by their normal counterparts, in agreement with a role for PEA1 in transformation by *raf*. Mutations in the ATP-binding site of v-*raf* prevented activation, suggesting that phosphorylation is somehow required.

Activation of transcription by mitogens appears to be mediated by a protein kinase cascade (14, 19). The *raf* gene family (A-, B-, and c-*raf*) encodes serine-threonine protein kinases which shuttle signals from the cellular to the nuclear membrane and possibly into the nucleus (U. R. Rapp, G. Heidecker, M. Huleihel, J. L. Cleveland, W. C. Choi, T. Pawson, J. N. Ihle, and W. B. Anderson, Cold Spring Harbor Symp Quant. Biol., in press). The transcription stimulation activity of the PEA1 motif (which is related to the AP1 consensus) (2, 9, 12) is increased by the expression of several non-nuclear oncogenes, serum components, and a tumor promoter (12-*O*-tetradecanoylphorbol-13-acetate) but not by the expression of nuclear oncogenes, except for *fos* (7, 21). We show here that the expression of *raf* oncogenes in vivo activates the PEA1 motif and that phosphorylation by *raf* is somehow involved in the activation process.

Stimulation of the activity of the PEA1 motif by v-*raf* expression. PEA1 transcription stimulation activity in LMTK$^-$ fibroblasts was measured by transfecting the reporter recombinants pG1PB4 and pG1PAB4 (Fig. 1A). pG1PB4 contains, upstream from the rabbit β-globin gene, four head-to-tail copies of the PEA1 motif from the polyomavirus enhancer. pG1PAB4 contains base-pair mutations which prevent specific binding of PEA1 in vitro (12). Cotransfection of 3611-MSV, which encodes v-*raf* (Fig. 1B), resulted in a 10-fold increase in PEA1 motif-dependent transcription (compare reporter bands for pG1PB4 and pG1PAB4 in lanes 1 to 4). To test whether the expression of v-*raf* oncoprotein was responsible for this increase, we used vectors J-1 and J-1a (Fig. 1B and reference 13). J-1 expresses a fusion protein between mouse v-*raf* and the highly homologous chicken v-*mil*, whereas J-1a contains a translation termination codon (T) in-frame with the *raf-mil* coding sequences. Transcription from the wild-type reporter was increased by cotransfection of J-1 (compare REPORTER bands in Fig. 2, lanes 5 and 3), whereas J-1a had no effect (compare REPORTER bands in Fig. 2, lanes 6 and 3), strongly suggesting that the v-*raf* oncoprotein stimulated PEA1 motif activity.

Inhibition of PEA1 motif activation by amino acid substitutions in the ATP-binding domain of v-*raf*. Comparisons of amino acid sequences between protein kinases suggest that Lys-53 of v-*raf* is directly involved in interactions with ATP (5a). We studied the effects of amino acid substitutions of Lys-53 and the adjacent Lys-56 on the ability of v-*raf* to activate the PEA1 motif (EH-282, EH-293, and EH-301 in Fig. 1B). The Lys-56→Gln substitution had a small inhibitory effect on *trans* activation; the Lys-56→Glu mutation was more inhibitory, whereas the Lys-53→Trp change abolished *trans* activation (compare lanes 7 to 10 and 3 in Fig. 2). These results strongly suggest that ATP binding and therefore also the kinase activity of v-*raf* are required for *trans* activation of the PEA1 motif.

Stimulation of the PEA1 motif only by oncogenically activated c-*raf*. Amino-terminal truncation renders c-*raf*-1 transforming and activates its kinase activity (5a; Rapp et al., in press). We tested whether amino-terminal truncation of c-*raf*-1 also affected PEA1 motif activation. The expression of full-length, nontransforming c-*raf*-1 did not affect PEA1 motif activity (Fig. 3, lanes 1 and 6, and Fig. 1B, EC). In contrast, the expression of truncated, transformation-competent c-*raf*-1 stimulated PEA1 motif activity about fourfold (Fig. 3, lanes 4 and 6, and Fig. 1B, EC12). A further truncated and nontransforming c-*raf*-1 did not activate PEA1 (Fig. 3, lanes 5 and 6, and Fig. 1B, EC13). These results show that amino-terminal truncation is sufficient to activate c-*raf*-1 in its capacity to stimulate the PEA1 motif.

Pertubation of the c-*raf*-1 protein by insertion of a tetrapeptide into a region which is serine-threonine rich and homologous to A-*raf* (EC2 and CR2 in Fig. 1B) produced a transforming oncoprotein. In contrast, insertion of a tetrapeptide into the ATP-binding domain (EC4 in Fig. 1B) did not activate *raf* (5a). Consistent with the transformation studies, we found that the transforming mutant activated the PEA1 motif (about fourfold; compare lanes 2 and 6 in Fig. 3), whereas the nontransforming mutant had little effect (less than twofold; compare lanes 3 and 6 in Fig. 3). These results show that a small insertion in CR2 of c-*raf*-1 can activate its potential to stimulate the PEA1 motif.

Stimulation of the PEA1 motif by the expression of activated A-*raf*. The expression of activated mouse or human A-*raf* stimulated PEA1 motif activity (compare lanes 11 and 12 with lane 3 in Fig. 2; Fig. 1B). In contrast, the expression of normal human A-*raf* was much less efficient in *trans* activation (compare lane 13 with lanes 11 and 12 in Fig. 2; Fig. 1B), although a small increase in PEA1 motif activity was ob-

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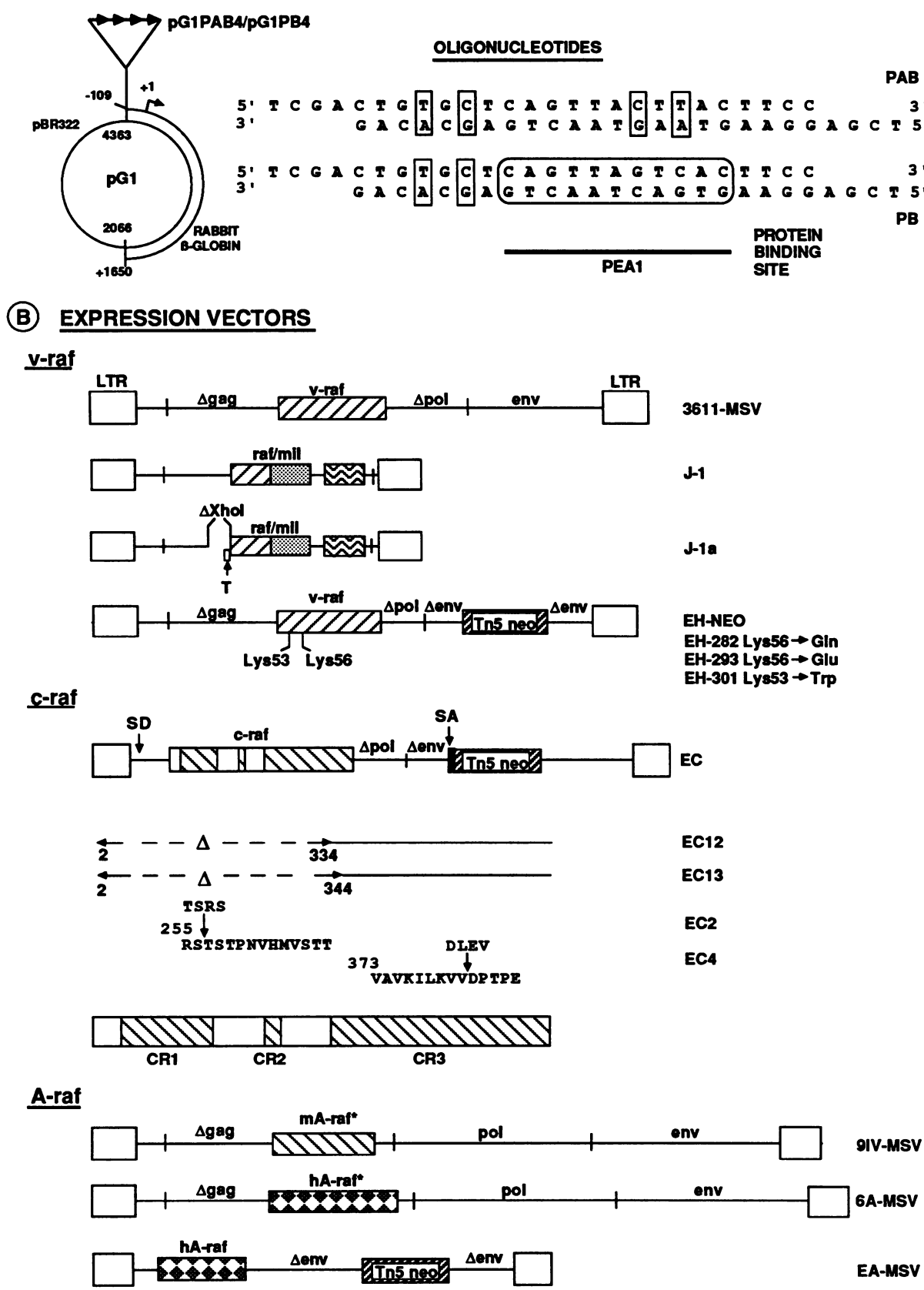
A REPORTERS
pG1PAB4/pG1PB4
-109
+1
pBR322
4363
pG1
2066
RABBIT
β-GLOBIN
+1650
OLIGONUCLEOTIDES
PAB
5' T C G A C T G T G C T C A G T T A C T T A C T T C C 3'
3' G A C A C G A G T C A A T G A A T G A A G G A G C T 5'
5' T C G A C T G T G C T C A G T T A G T C A C T T C C 3'
3' G A C A C G A G T C A A T C A G T G A A G G A G C T 5'
PB
PEA1
PROTEIN
BINDING
SITE
B EXPRESSION VECTORS
v-raf
LTR
Δgag
v-raf
Δpol
env
LTR
3611-MSV
raf/mil
J-1
ΔXhoI
raf/mil
T
J-1a
Δgag
v-raf
Δpol
Δenv
Tn5 neo
Δenv
Lys53
Lys56
EH-NEO
EH-282 Lys56→Gln
EH-293 Lys56→Glu
EH-301 Lys53→Trp
c-raf
SD
c-raf
SA
Δpol
Δenv
Tn5 neo
EC
Δ
2
334
EC12
Δ
2
344
EC13
TSRS
255
RSTSTPNVHMVSTT
EC2
373
VAVKILKVVDPTPE
DLEV
EC4
CR1
CR2
CR3
A-raf
Δgag
mA-raf*
pol
env
9IV-MSV
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hA-raf*
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env
6A-MSV
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Δenv
EA-MSV

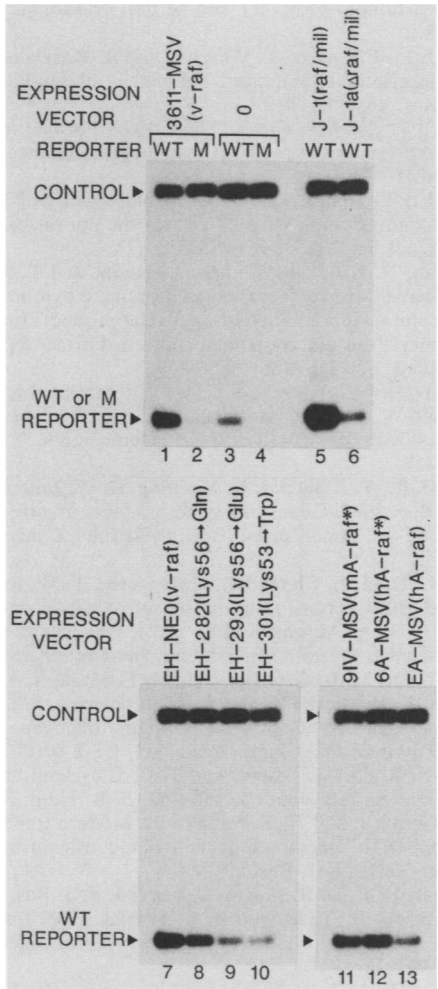

FIG. 2. Effects of v-*raf* and A-raf expression on PEA1 motif activity in LMTK$^-$ fibroblasts. LMTK$^-$ cells were transfected with 1 μg of reporter recombinant (pG1PB4 or pG1PAB4), 1 μg of internal control (pβCB×2; see reference 21), and 5 μg of either the indicated expression vectors or pEMBL (expression vector 0). After transfection, the cells were washed and incubated for 48 h in medium containing 0.05% fetal calf serum, and total RNA was extracted and analyzed by quantitative S1 nuclease mapping (for experimental details, see references 7 and 20–22). The bands labeled WT or M REPORTER or CONTROL were the products resulting from S1 nuclease analysis and derived from RNA specifically initiated from the globin and conalbumin promoters of the corresponding recombinants. The results were derived from one representative transfection, and the band intensities can be compared among all the lanes. WT, Wild type; M, mutant.

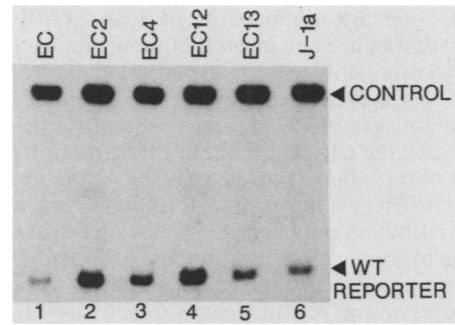

FIG. 3. Effect of c-*raf* expression on PEA1 motif activity in LMTK$^-$ cells. LMTK$^-$ fibroblasts were transfected as described in the legend to Fig. 2 with the indicated *raf* expression vectors. For CONTROL or WT REPORTER, see the legend to Fig. 2.

served (about twofold; compare lanes 13 and 3; results not shown). Thus, oncogenic activation of A-*raf* increases its ability to activate the PEA1 motif.

We have shown that the expression of c-*raf*-1 and A-*raf* proteins, which are encoded by different genes and whose properties differ in several ways (5a), activates a common target, the PEA1 motif. Different members of the *ras* family (Ha-*ras*, Ki-*ras*, and N-*ras*) also activate the PEA1 motif (A. Pahler and B. Wasylyk, unpublished results), showing that there is a common DNA target for different members of both oncogene families. We have detected only one component (PEA1) in LMTK$^-$ fibroblast extracts which interacts with this motif (7), suggesting that it is the target for oncogene activation of the PEA1 motif.

In addition to *raf*, the expression of v-*src*, polyomavirus middle T, *ras*, v-*mos*, c-*fos*, serum ingredients, and the tumor promoter TPA activates the PEA1 motif (21). In contrast, the expression of several other oncogenes does not (v-*myc*, simian virus 40 large T, polyomavirus large T, and adenovirus EIA) (21). The activation of *raf* protein kinase could be one of the later elements of a cascade of events which links mitogens and the PEA1 motif-activating oncogenes to an effect in the nucleus. Stimulation of cells with mitogens leads to a redistribution of c-*raf* to the nuclear area (Rapp et al., in press). A variety of mitogenic signals and membrane-bound oncogene products increase c-*raf*-1 phosphorylation and an associated protein kinase activity (11). Interestingly, all of these that we have tested also activate the PEA1 motif (v-*src*, polyomavirus middle T, *ras*, TPA, and serum growth factors).

Phosphorylation by *raf* is directly or indirectly important for the activation of transcription, since mutations in the ATP-binding site of v-*raf* prevent *trans* activation. It is possible that *raf* directly phosphorylates a nuclear protein.

FIG. 1. Structures of recombinants. (A) Reporter recombinants. The reporter recombinants pG1PAB4 and pG1PB4 contain four head-to-tail copies of the PAB and PB oligonucleotides. Both oligonucleotides contain a 2-base-pair mutation which prevents binding of the PEA2 factor (see boxed nucleotides common to both PB and PAB and reference 12). The PAB oligonucleotide contains, in addition, a two-base-pair mutation which prevents the specific binding of PEA1 (see additional boxed nucleotides and reference 12). (B) Expression vectors. The expression vectors contain retrovirus MSV sequences in pBR322. Top maps show v-*raf* vectors. 3611-MSV expresses v-*raf* as a fusion protein with *gag*. J-1 produces a fusion protein between the homologous mouse v-*raf* and chicken v-*mil* oncoproteins. J-1a contains a deletion of an *Xho*I restriction fragment (ΔXhoI) which introduces a termination codon (T) in front of the reading frame for *raf-mil*. EH-282, EH-293, and EH-301 are derived from EH-NE0 by site-directed mutagenesis and code for the indicated changes in the protein sequence of v-*raf* at either Lys-56 or Lys-53. Middle maps show c-*raf*-1 vectors. EC expresses human c-*raf*-1, whereas EC12 and EC13 express amino-terminally truncated *raf* proteins. EC2 and EC4 express proteins with four-amino-acid insertions in either conserved region 2 (CR2) or conserved region 3 (CR3). SD, Splice donor; SA, splice acceptor. Bottom maps show A-*raf* vectors. 91V-MSV codes for a fusion protein between *gag* and mouse A-*raf* (mA-raf*). 6A-MSV codes for a *gag*-human A-*raf* oncoprotein (hA-raf*). EA-MSV expresses normal human A-*raf* protein without *gag* sequences (hA-raf). For further descriptions, see references 3, 5a, 6, and 13–16; and Rapp et al., in press).

c-*fos* and c-*jun* are components of PEA1 (AP1; 17, 18), suggesting that either one or both of them may be targets for phosphorylation. However, posttranslational modification of proteins is not sufficient to explain all aspects of the mechanism of activation of PEA1, since protein synthesis inhibitors can block the activation of the PEA1 motif by TPA (8).

Several observations suggest that the activation of PEA1 is an important event in transformation. Its activity is up-regulated by six oncogenes and down-regulated by EIA expression in some cell types (unpublished results). There is a good correlation between the capacities of various derivatives of the *raf* and *ras* oncogenes to activate PEA1 and to transform cells (this work; 21). Finally, two of the components of PEA1, v-*jun* (1, 4, 5, 8, 10) and *fos*, are oncogenes. Abnormal regulation of PEA1 activity could result in the altered expression of transformation-related genes.

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