

## Activation of the Mitogen-Activated Protein Kinase Pathway Induces Transcription of the *PAC-1* Phosphatase Gene

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***PAC-1*, an early-response gene originally identified in activated T cells, encodes a dual-specificity mitogen-activated protein kinase phosphatase. Here we report on the regulation of *PAC-1* expression in murine hemopoietic cells. *PAC-1* mRNA levels rapidly increase in mitogen-stimulated lymphocytes, with the induced expression being transient in B cells but sustained in activated T cells. Transfection analysis of murine *PAC-1* promoter-reporter constructs established that in T cells, sequences necessary for basal and induced transcription reside within a 200-bp region located immediately upstream of the transcription initiation sites. Basal transcription is regulated in part by an E-box element that binds a 53-kDa protein. *PAC-1* transcription induced by phorbol myristate acetate stimulation and the expression of the *v-ras* or *v-raf* oncogene is mediated via the E-box motif and an AP-2-related site and coincides with increased binding activity of the constitutive 53-kDa E-box-binding protein and induced binding of AP-2. The ability of an interfering ERK-2 mutant to block phorbol myristate acetate and *v-ras*-dependent *PAC-1* transcription indicates that mitogen-activated protein kinase activation is necessary for these stimuli to induce transcription of the *PAC-1* gene in T cells.**

A group of conserved serine/threonine kinases collectively referred to as mitogen-activated protein (MAP) kinases, extracellular signal-regulated (ERK) kinases, and stress-activated protein kinases participate in responses to a wide variety of extracellular stimuli (4, 7, 9, 24, 33, 34, 41, 46). MAP kinases are activated by phosphorylation of the threonine and tyrosine residues in the conserved motif Thr-X-Tyr (X = glycine, proline, or glutamic acid) by the dual-specificity MEK or MAP kinase kinases (24, 33, 34, 41, 46). The activated MAP kinases, in turn, serve to regulate a range of cellular processes including proliferation and differentiation by phosphorylating a diverse array of proteins. In the cytoplasm, these substrates include other kinases such as ribosomal S6 protein kinases (4, 9), phosphatases (44), growth factor receptors (9, 24), and cytoskeletal proteins (4, 9, 24). MAP kinases are also translocated to the nucleus (6) and regulate gene expression by phosphorylating transcription factors such as Elk-1 (19, 20, 51), c-Jun (11), and ATF-2 (18).

MAP kinases can be inactivated by dephosphorylation of the threonine or tyrosine residues in the conserved Thr-X-Tyr motif (1, 2, 10, 40, 49, 53, 54). While a number of phosphatases such as protein phosphatase 2A have been implicated in the inactivation of ERK-2 in a variety of cell types (1), a family of nuclear phosphatases related to the vaccinia virus dual-specificity phosphatase VH1 (16) have recently emerged as important regulators of MAP kinase function. These phosphatases, while possessing the canonical sequence HCXAGXXR(S/T), common to all protein tyrosine phosphatases (10), exhibit catalytic activity for both phosphotyrosine and phosphothreonine in MAP kinase substrates (2, 10, 17, 23, 37, 49, 53). To date, five distinct mammalian VH1-like MAP kinase phosphatases have been identified (2, 5, 17, 23, 37, 42, 48). These phosphatases share a conserved carboxyl terminus encompassing the

catalytic domain (54), while the amino termini of the various family members exhibit considerable sequence divergence. Constitutive expression of these phosphatases in different tissues (5, 17, 23, 29, 37, 42, 48), coupled with differential expression induced in response to a variety of stimuli in a number of cell types (5, 23, 27, 29, 37, 48), suggests that each phosphatase serves a distinct role in the regulation MAP kinases. One member of the MAP kinase phosphatase family, *PAC-1*, originally identified as the nuclear product of an early-response gene induced during T-cell activation (48), appears to be expressed solely in hemopoietic tissues (48, 54). Consistent with its homology to other dual-specificity phosphatases, *PAC-1* exhibits stringent substrate specificity for MAP kinases in vitro, and constitutive *PAC-1* expression in T cells downregulates ERK-2 activity (53, 54).

To further understand the role of *PAC-1*, we have examined the regulation of *PAC-1* mRNA expression in different murine hemopoietic cells. While *PAC-1* mRNA levels rapidly increase in lymphocytes activated by a variety of mitogenic stimuli, the duration of induced expression differs in B and T cells. Transcriptional activation of the *PAC-1* promoter in T cells in response to *v-ras*- and *v-raf*-induced signals, is mediated by AP-2 and an E-box-binding protein and indicates that *PAC-1* transcription is induced in response to the activation of the MAP kinase cascade.

### MATERIALS AND METHODS

**Cell culture.** B-lymphoid cell lines were cultured in RPMI 1640–10% fetal calf serum (FCS; Flow Laboratories)–50  $\mu$ M 2-mercaptoethanol (2-ME; Sigma, St. Louis, Mo.). The Jurkat T-cell line was maintained in RPMI 1640–8% FCS, and p41.1 T cells were grown in RPMI 1640–10% FCS–50  $\mu$ M 2-ME–10 U of interleukin-2 (IL-2) per ml. Concanavalin A (ConA; Pharmacia) (5  $\mu$ g/ml) or 12-*O*-tetradecanoylphorbol-13-acetate (PMA; 75 ng/ml; Sigma) and ionomycin (1  $\mu$ g/ml; Sigma) stimulations of p41.1 cells were done in RPMI 1640–1% FCS–50  $\mu$ M 2-ME for the indicated times. Following the transient transfection of Jurkat cells with various expression and reporter gene plasmids, all cells were incubated for 48 h prior to the preparation of cell extracts for chloramphenicol acetyltransferase (CAT) assays. Transfected cells treated with PMA (75 ng/ml) were stimulated for 4 h prior to the generation of cell extracts. The progranulocyte/macrophage cell line SPGM1 was maintained as described previously (35)

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and allowed to differentiate to myelomonocytic cells by growth in medium containing a supramaximal concentration of murine IL-3. The M1 cell line was maintained in Dulbecco's modified Eagle medium containing 10% FCS. Separate cultures containing  $2 \times 10^4$  to  $10 \times 10^5$  M1 cells per ml were stimulated with murine leukemia inhibitory factor (LIF; 4 ng/ml; AMRAD, Kew, Victoria, Australia) or murine IL-6 (32 ng/ml; PeproTech) for each time point as previously described (50). Cytochrome preparations were stained with May-Grunwald-Giemsa stain to assess the extent of macrophage differentiation. All fibroblasts were grown in Dulbecco's modified Eagle's medium containing 5% FCS.

Splenic B and T cells ( $10^6$ /ml) from 6- to 8-week old C57BL/6 mice were purified and cultured as described previously (28). B lymphocytes were stimulated with either lipopolysaccharide (LPS; Difco) at a concentration of 20  $\mu$ g/ml, recombinant chimeric mouse CD40 ligand-CD8a fusion protein (30) at 2  $\mu$ g/ml, or affinity-purified goat anti-mouse immunoglobulin (IgM) (Fab')<sub>2</sub> fragments (Cappel) at 20  $\mu$ g/ml. T lymphocytes were stimulated with ConA at 2  $\mu$ g/ml or with a combination of PMA (2 ng/ml) and ionomycin (1  $\mu$ g/ml).

**RNase protection mapping.** Ten-microgram samples of total cytoplasmic RNA isolated from murine cell lines or primary cells were hybridized to 165- and 150-nucleotide [ $\alpha$ -<sup>32</sup>P]UTP-labeled antisense RNA probes which encompass nucleotides 450 to 615 of the murine *PAC-1* mRNA (48) and part of the rat *GAPDH* mRNA, respectively. RNase A and RNase T<sub>1</sub> digestions were performed as described previously (14), and the products were analyzed on 5% acrylamide-8 M urea gels.

**Plasmid constructs.** Plasmids N/N-CAT, K/N-CAT, XbaI/N-CAT, NarI/N-CAT, and XhoI/N-CAT consisted of the 3.0-kb *Nco*I, 1.7-kb *Kpn*I-*Nco*I, 324-bp *Xba*I-*Nco*I, 169-bp *Nar*I-*Nco*I, and 125-bp *Xho*I-*Nco*I genomic fragments, respectively, from the 5' flanking sequence of the murine *PAC-1* gene, inserted upstream of the CAT gene in the promoterless reporter plasmid pBLCAT3 (31). *Xba*I/N.E<sub>m</sub>-CAT and *Xba*I/N.AP-2<sub>m</sub>-CAT are derivatives of plasmid *Xba*I/N-CAT in which the E-box motif (CACGTG) and the AP-2-related site (GGC CAGGC) at positions -108 to -113 and -52 to -45, respectively, were altered by in vitro mutagenesis (21) to 5'-GGTGTG-3' (E box) and 5'-TGCCATTC-3' (AP-2). Ewt.TK-CAT and AP-2.TK-CAT were generated by inserting oligonucleotides encompassing the *PAC-1* E box (5'-GTCCCACGTGAAGC-3') and AP-2-like (5'-CGAGGGCCAGGCTCG-3') motifs (underlined) upstream of the thymidine kinase (TK) promoter in the reporter plasmid pBLCAT-2 (31). Plasmids Emut.TK-CAT and AP-2 mut.TK-CAT have mutant E-box (5'-GTCCG GTGTGAAGC-3') and AP-2-like (CGAGTGCCATTCTCG-3') motifs (underlined) inserted in pBLCAT-2. The *v-Ha-ras* and *v-raf* expression vectors pEFbos.*v-ras* and pEFbos.*v-raf* were kind gifts of D. Cantrell (Imperial Cancer Research Foundation, London, England). Plasmid pEF-PGK*puro.v-ras* was prepared by inserting a 0.6-kb *Bam*HI-*Xba*I fragment encoding *v-ras* into the puromycin resistance plasmid pEF-PGK*puro* (a kind gift of David Huang, Walter and Eliza Hall Institute). The ERK-2 mutant ERK-2<sub>m</sub>, in which the threonine and tyrosine residues at positions 183 and 185 were changed to alanine and phenylalanine, respectively, and the *v-Ha-ras* mutant G26, in which the asparagine residue at position 26 was changed to glycine, were generated by PCR-based in vitro mutagenesis (21). ERK-2<sub>m</sub> and *v-ras* G26 were inserted into pEFbos and pEF-PGK*puro*, respectively.

**Transfections and CAT assays.** Jurkat cells with stably integrated copies of the various *PAC-1* promoter-CAT reporter plasmids were generated by coelectroporating (250 V, 960  $\mu$ F) 10<sup>7</sup> Jurkat cells with linearized pSV2Neo (2  $\mu$ g) and a 10-fold molar excess of *PAC-1* promoter-CAT plasmid (20 to 30  $\mu$ g). Electroporated cells ( $3 \times 10^5$ /ml) were incubated for 48 h and then subjected to selection in Geneticin (800  $\mu$ g/ml; Gibco, BRL) for a further 18 days. Dot blots on genomic DNA isolated from G418-resistant cells were used to confirm the presence of integrated CAT plasmid in the pooled transfectants. G418-resistant cells were routinely passaged in 200  $\mu$ g of geneticin per ml to ensure continued selection of the integrated plasmids. Jurkat cells stably transfected with 30  $\mu$ g of linearized pEF-PGK*puro*, pEF-PGK*puro.v-ras*, or pEF-PGK*puro.v-ras*G26 were generated as described above. Electroporated cells ( $2.5 \times 10^5$ /ml) were incubated for 48 h in 2-ml cultures of RPMI 1640-10% FCS and then subjected to selection in puromycin (3  $\mu$ g/ml; Sigma). Ten days later, puromycin-resistant cells from individual wells were cloned by limit dilution. Cells transfected with pEF-PGK*puro.v-ras* or pEF-PGK*puro.v-ras*G26 were checked for expression of the transfected *v-ras* gene by RNase protection and Western blotting (immunoblotting). Clones expressing similar levels of *v-ras* and *v-ras* G26 were subsequently used for all experiments.

For all transient transfections with murine *PAC-1* promoter-CAT reporter gene plasmids, approximately 10<sup>7</sup> cells were transfected by using a modification of the DEAE-dextran method (52). Expression vectors for *v-ras* and ERK-2<sub>m</sub> were cotransfected with reporter plasmids at a threefold molar excess. Jurkat cells stably transfected with *PAC-1* promoter-CAT plasmids were transiently transfected with equimolar amounts (10 to 12  $\mu$ g) of pEFbos, pEFbos.*v-ras*, pEFbos.*v-raf*, or pEFbos.ERK-2<sub>m</sub>. Approximately 48 h posttransfection, cells were harvested and extracts were prepared as described previously (15). Cellular extracts were standardized for protein content, and CAT assays were performed with 15  $\mu$ g of protein. Reaction products were then subjected to thin-layer chromatography, and the fraction of acetylated chloramphenicol was determined by PhosphorImager (Molecular Dynamics) analysis. Each series of transfections was performed four or five times, with a maximum variance of approximately 15% observed between replicate experiments.

**EMSA and UV cross-linking.** The preparation of nuclear extracts and the conditions used for electrophoretic mobility shift assays (EMSA) have been described previously (14, 15). The following gel-purified <sup>32</sup>P-labeled double-stranded oligonucleotides (specified sequences are underlined) were used as probes in DNA binding reactions with 3  $\mu$ g of nuclear extract: Ewt (5'-GCCA GAGTCCCACGTGAAGCCGC-3'), the E-box site from the murine *PAC-1* promoter (12); Emut (5'-GCCAGAGTCCGGTGTGAAGCCGC-3'), a mutant version of Ewt; Inwt (5'-GGCGCCTTCTGCTTACCCTTGTATGGTGAC CCGAGGGCCAGGCTCGAG-3'), encompassing nucleotides -89 to -40 of the murine *PAC-1* promoter; InAP-2<sub>m</sub> (5'-GGCGCCTTCTGCTTACCCTTGTATGGTGACCGAGTCCCGAGTGCATTCTCGAG-3'), a version of Inwt in which the putative AP-2 site has been mutated; and 5'-TCGGGGCGGGG-3', which encompasses a consensus binding site for the Sp1 transcription factor. One nanogram of recombinant human AP-2 (Promega, Madison, Wis.) was used for gel shifts with Inwt and InAP-2<sub>m</sub> probes, while  $\alpha$  supershifts were performed with anti-AP-2 serum (Santa Cruz Biotechnology, Santa Cruz, Calif.).

The cross-linking method has been described previously (14). Labeled probe was made by first annealing the oligonucleotide 5'-CCAGCCAGAGTCCACGTGAAGCCGCGGGG-3', spanning the E-box site in the murine *PAC-1* promoter, to the complementary 10-mer 5'-CCCCCGCGG-3'. Radiolabeling was performed with the Klenow fragment of DNA polymerase I in the presence of 10 mM Tris-Cl (pH 7.4), 6 mM MgCl<sub>2</sub>, 50 mM NaCl, 6 mM 2-ME, 0.2 mg of gelatin per ml, 20  $\mu$ M bromo-dUTP, 20  $\mu$ M each dATP and GTP, and 100  $\mu$ Ci each of [ $\alpha$ -<sup>32</sup>P]dATP and [ $\alpha$ -<sup>32</sup>P]dCTP. Approximately 10<sup>6</sup> cpm of gel-purified probe was incubated with 10  $\mu$ g of nuclear extract, and protein-DNA complexes were electrophoresed by using a standard EMSA protocol. Gels were then covered with plastic wrap, irradiated on a UV transilluminator (302 nm) for 1 h, and exposed to X-ray film. The complexes were eluted from gel slices in 3% sodium dodecyl sulfate (SDS), and the protein-DNA adducts were resolved on SDS-10% polyacrylamide gels.

## RESULTS

***PAC-1* gene expression is rapidly induced in B and T lymphocytes by different stimuli.** While it has been established that the expression of human *PAC-1* is largely confined to tissues such as the spleen and thymus (48), little is known about *PAC-1* expression in different types of hemopoietic cells. Consequently, the expression of murine *PAC-1* mRNA was examined by RNase protection in hemopoietic cell lines and normal B and T cells treated with various stimuli (Fig. 1).

We first investigated the expression of *PAC-1* in cell lines representing various stages of differentiation within the B-cell lineage (Fig. 1A, upper panel). *PAC-1* mRNA was detected in all pre-B- and B-cell lines (lanes 2 to 8) but not in plasmacytomas (lanes 9 to 11), indicating that this phosphatase may be expressed in a differentiation stage-specific manner during B-cell development. In contrast to cell lines, *PAC-1* mRNA was not detected in resting splenic B cells (Fig. 1A, lower panel, lane 3). However, mitogenic stimulation of primary B cells (Fig. 1A, lower panel) with optimal concentrations of LPS (lanes 4 to 8), CD40 ligand (lanes 9 to 13), or antibodies specific for the IgM receptor (lanes 14 to 18) rapidly induced *PAC-1* expression. *PAC-1* mRNA was induced within 30 min in LPS- and anti-IgM-treated cells, whereas CD40 ligand-induced expression was first detected 1 h postactivation. For all of the B-cell mitogen stimulations, *PAC-1* expression peaked at 4 h poststimulation but had declined considerably by 24 h. These findings indicate that in normal B cells, *PAC-1* is an early-response gene which is expressed in a transient manner.

*PAC-1* transcription has been shown previously to be rapidly induced in the Jurkat T-cell line following mitogenic stimulation (48). Consistent with these findings, we demonstrated that *PAC-1* mRNA was induced by mitogens in murine T cells (Fig. 1B). In both the T-cell line p41.1 (13) (upper panel, lanes 3 to 8) and normal splenic T cells (lower panel, lanes 3 to 8), the phorbol ester PMA in combination with the calcium ionophore ionomycin rapidly induced the expression of *PAC-1* mRNA with similar and sustained kinetics over a 24-h period. ConA-dependent induction of *PAC-1* expression in p41.1 cells was similar to that seen with PMA and ionomycin treatment (upper panel, lanes 9 to 15). However, in normal T cells, only a modest

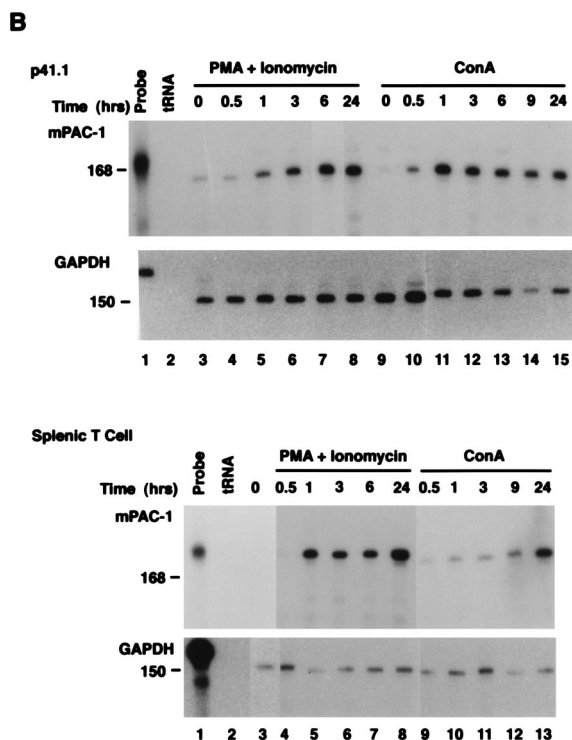
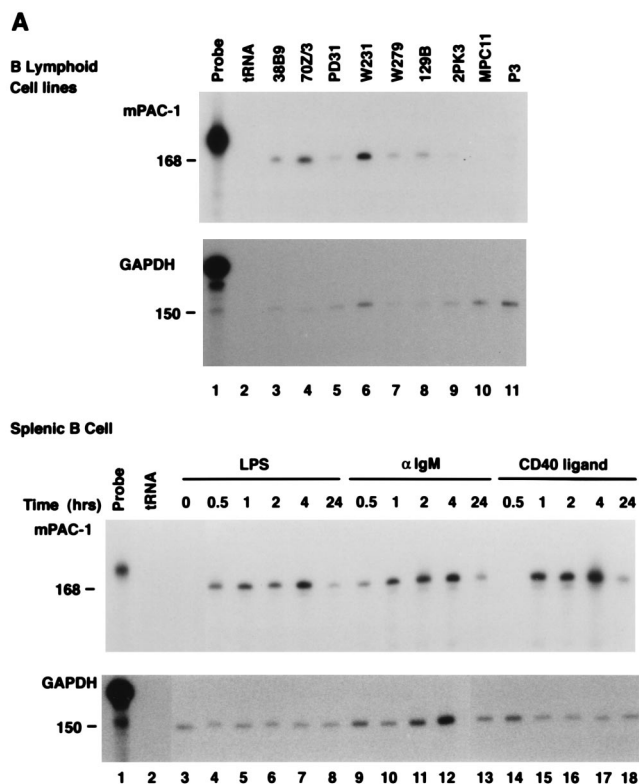
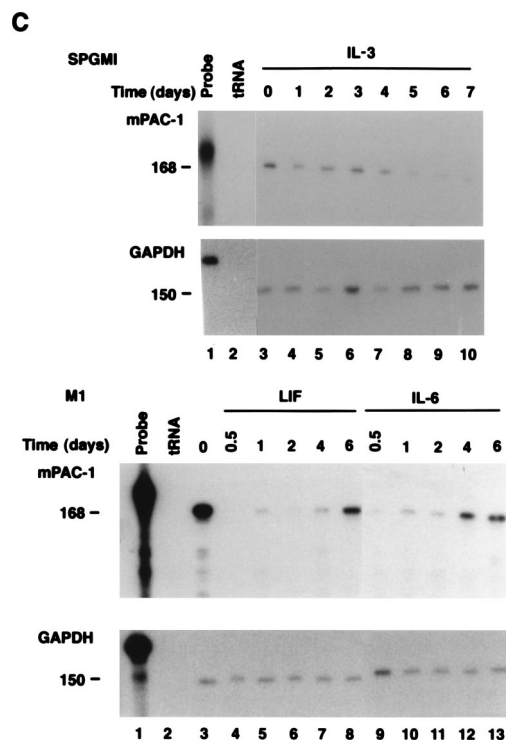


FIG. 1. Analysis of murine *PAC-1* expression in hemopoietic cells. Ten-microgram samples of total cytoplasmic RNA isolated from B-cell lines and splenic B cells were hybridized to the 168-nucleotide murine *PAC-1* and 150-nucleotide rat *GAPDH* probes, digested with RNases A and T<sub>1</sub>, and then fractionated on 5% acrylamide-urea gels. The gels were exposed for 24 h of autoradiography at -70°C. The sizes of the protected products are indicated in nucleotides to the left of each panel. (A) Constitutive and induced murine *PAC-1* expression in B-lineage cells. In the upper panel, total cytoplasmic RNA was isolated from the following cells lines: 38B9, 70Z/3 and PD31 (pre B-cell lymphomas) (lanes 3 to 5); W231, W279, and 129B (B-cell lymphomas) (lanes 6 to 8); and 2PK3, MPC11, and P3 (plasmacytomas) (lanes 9 to 11). In the lower panel, total cytoplasmic RNA was isolated from resting splenic B cells (lane 3) or splenic B cells stimulated with LPS (lanes 4 to 8), anti-IgM (αIgM; lanes 9 to 13), and CD40 ligand (lanes 14 to 18) for the indicated times. (B) Murine *PAC-1* expression is induced during T-cell activation. In the upper panel, total cytoplasmic RNA isolated from the T-cell line p41.1 prior to stimulation (lanes 3 and 9) or after treatment with PMA-ionomycin (lanes 4 to 8), or ConA (lanes 10 to 15) for the indicated times was subjected to RNase protection analysis. In the lower panel, RNase protection analysis was performed on total cytoplasmic RNA isolated from splenic T cells before (lane 3) or after incubation for the indicated times with PMA-ionomycin (lanes 4 to 8) and ConA (lanes 9 to 13). (C) Murine *PAC-1* expression changes during hemopoietic differentiation. In the upper panel, total cytoplasmic RNA was isolated from the SPGM1 cell line prior to (lane 3) or at the indicated times during IL-3-mediated myelomonocytic differentiation (lanes 4 to 10). In the lower panel, total cytoplasmic RNA isolated from untreated M1 cells (lane 3) or M1 cells stimulated with LIF (lane 4 to 8) or IL-6 (9 to 13) for the indicated times was subjected to RNase protection.



increase in *PAC-1* mRNA was observed up to 9 h poststimulation, with peak levels of expression reached only after extended stimulation (lower panel, lanes 9 to 13). These findings indicate that the kinetics of mitogen-induced *PAC-1* expression

in T-cell lines and primary T lymphocytes depends on the type of stimulus, and unlike the case for B cells, elevated expression of *PAC-1* mRNA persists for at least 24 h poststimulation.

Since *PAC-1* transcription increases during lymphocyte ac-

tivation, we decided to examine whether expression also changes during hemopoietic differentiation. This was investigated in two different cell line models. The SPGM1 cell line normally exhibits properties of CD5-positive pre-B cells (35). When treated with IL-3, SPGM1 differentiates over a 4- to 5-day period into cells with characteristics of a macrophage/granulocyte (35). Like other pre-B-cell lines, untreated SPGM1 expresses *PAC-1* mRNA (Fig. 1C, upper panel, lane 3). However, after IL-3 stimulation, *PAC-1* levels in SPGM1 decreased by day 1 and virtually disappeared by day 5. This reduced expression coincided with the differentiation of almost the entire SPGM1 population. In the second model, treatment of the M1 monocytic precursor with LIF or IL-6 induced terminal monocyte/macrophage differentiation over a 6-day period (50). Whereas *PAC-1* expression in M1 cells is downregulated within 12 h of cytokine treatment (Fig. 1C, lower panel, lanes 4 and 9), it increases again by day 6 (lanes 8 and 13) when the process of differentiation is complete. The role of biphasic *PAC-1* expression during IL-6- and LIF-induced M1 differentiation remains to be determined.

**Basal and v-Ras- or v-Raf-induced *PAC-1* transcription requires an E-box motif.** To identify regulatory elements necessary for the control of murine *PAC-1* transcription, a nested set of restriction fragments extending upstream of the *PAC-1* initiation codon (Fig. 2A) was inserted 5' of the CAT gene in the promoterless reporter plasmid pBLCAT3 (31) (here designated pCAT) and transfected stably into the human T-cell line Jurkat. Plasmid N/N-CAT, which encompasses approximately 2,900 bp of 5' flanking sequence, exhibited significantly higher promoter activity than the parental vector (Fig. 2B; compare lanes 1 and 5). Flanking sequence deletions to a point 244 bp upstream of the major site of transcription initiation exhibited promoter activity similar to that of the full-length N/N-CAT plasmid (lanes 5, 9, and 13). However, a further truncation of 155 nucleotides (NarI/N-CAT; 89 nucleotides upstream of the major transcription start site) reduced basal promoter activity approximately twofold compared with N/N-CAT (compare lanes 5 and 17), while plasmid XhoI/N-CAT, which has only 40 bp of sequence upstream of the major transcription initiation site, exhibited the same promoter activity as pCAT3 (lane 21). This result indicated that sequences essential for basal promoter activity reside between nucleotides -244 and -89.

Scrutiny of the nucleotide sequence between -244 and -89 revealed a consensus E-box motif (39) that is conserved within an equivalent region of the murine *MKP-1* promoter (42). To determine if the E-box element was necessary for basal *PAC-1* transcription in T cells, the promoter activity of XbaI/N-CAT in which the E-box was mutated (XbaI/N.E<sub>m</sub>-CAT) was compared with that of the parental plasmid. The finding that the activity of XbaI/N.E<sub>m</sub>-CAT (Fig. 2C, lane 12) was reduced to that of NarI/N-CAT established that the E-box element and sequences that reside between -89 and -40 are required for basal *PAC-1* transcription in T cells.

To identify the region of the *PAC-1* promoter involved in mitogen-dependent transcription, PMA, an agent that induces *PAC-1* transcription in Jurkat cells (26, 48), was used to stimulate cells that had been stably transfected with various *PAC-1* reporter constructs. Whereas PMA upregulated N/N-CAT, K/N-CAT, and XbaI/N-CAT transcription between two- and threefold (Fig. 2B, lanes 6, 10, and 14), no phorbol ester-induced transcription of XhoI/N-CAT was observed (lane 22). However, a threefold elevation of NarI/N-CAT activity following stimulation by PMA (lane 18) indicated that the region of the *PAC-1* promoter required for phorbol ester-dependent transcription resided between nucleotides -89 and -40. Since *PAC-1* expression induced by PMA and other extracellular

signals also activated MAP kinase, we examined whether *PAC-1* promoter activity was induced by the same signal transduction pathway that activates the MAP kinases ERK-1 and ERK-2. As v-Ras and v-Raf are constitutively activated viral homologs of cellular proteins involved in the MAP kinase signaling cascade (3), these oncoproteins were tested for the ability to increase *PAC-1* transcription. Jurkat cells with integrated N/N-CAT, K/N-CAT, XbaI/N-CAT, NarI/N-CAT, and XhoI/N-CAT plasmids were transfected with *v-ras* (Fig. 2B, lanes 7, 11, 15, 19, and 23) or *v-raf* (lanes 8, 12, 16, 20, and 24) expression vectors. All plasmids except XhoI/N-CAT displayed enhanced transcription. The inability of these oncoproteins to upregulate XhoI/N-CAT indicated that the v-Ras- or v-Raf-responsive region of the promoter, like the PMA-inducible element, was located between nucleotides -244 and -40. However the finding that v-Ras or v-Raf upregulated NarI/N-CAT transcription only two- to threefold (lanes 19 and 20) rather than by approximately fivefold as observed for XbaI/N-CAT indicated that the v-Ras- or v-Raf-induced transcriptional response appeared to be mediated via multiple distinct regulatory sequences located between -244 and -40. The requirement of the E box for basal *PAC-1* transcription prompted an examination of whether this element was also involved in v-Ras- or v-Raf-dependent transcription. Consistent with the diminished transactivation of NarI/N-CAT, XbaI/N.E<sub>m</sub>-CAT expression was upregulated only approximately twofold (Fig. 2C, lane 15) upon *v-ras* cotransfection. This result establishes that while the E box is required for v-Ras- or v-Raf-dependent transcription, a distinct element residing between -88 and -40 also contributes to *PAC-1* transcription mediated by these oncoproteins.

Although v-Ras and v-Raf activate the MAP kinase signaling cascade, it remained a formal possibility that *PAC-1* transcription mediated by these oncoproteins involved other distinct signaling pathways. To test if PMA- and v-Ras-induced *PAC-1* transcription in Jurkat cells was dependent on MAP kinase activation, cells activated by these stimuli were also transiently cotransfected with an interfering ERK-2 mutant (ERK-2<sub>m</sub>) that disrupts MAP kinase signals (8). Consistent with a model in which these stimuli activate MAP kinase-dependent *PAC-1* transcription, PMA (Fig. 2C, lane 11)- and v-Ras (lane 12)-dependent XbaI/N-CAT activity was reduced to near basal levels by coexpression of ERK-2<sub>m</sub>. Interestingly, ERK-2<sub>m</sub> does not significantly reduce basal XbaI/N-CAT transcription (Fig. 2C, lane 10), suggesting that it is not regulated by ERK-2-dependent signals.

To further establish the role of the E box in v-Ras- or MAP kinase-dependent *PAC-1* transcription, wild-type (Ewt) and mutant (Emut) E-box elements were inserted upstream of the heterologous TK promoter in the reporter plasmid pBLCAT-2 (31) (here designated TK-CAT) and transiently cotransfected with pEFbos.v-ras, pEFbos.ERK-2<sub>m</sub>, or both expression plasmids into Jurkat cells (Fig. 3). Ewt.TK-CAT transcription, upregulated approximately threefold by v-Ras (lane 6), was suppressed when v-Ras was coexpressed with ERK-2<sub>m</sub> (lane 8). Consistent with results obtained with XbaI/N.Emut-CAT (Fig. 2B, lane 15), Emut.TK-CAT transcription was not upregulated by v-Ras (Fig. 3, lane 10). Collectively, these results indicate that the E-box element is required for v-Ras-induced ERK-2-dependent *PAC-1* transcription in T cells.

**v-Ras- or v-Raf-inducible complexes bind to E-box and AP-2 motifs in the murine *PAC-1* promoter.** The protein binding properties of sequences involved in murine *PAC-1* transcription were investigated by using EMSAs. Probes encompassing the *PAC-1* E-box motif (Ewt) or a mutated version (Emut) were incubated with nuclear extracts isolated from Jurkat cells

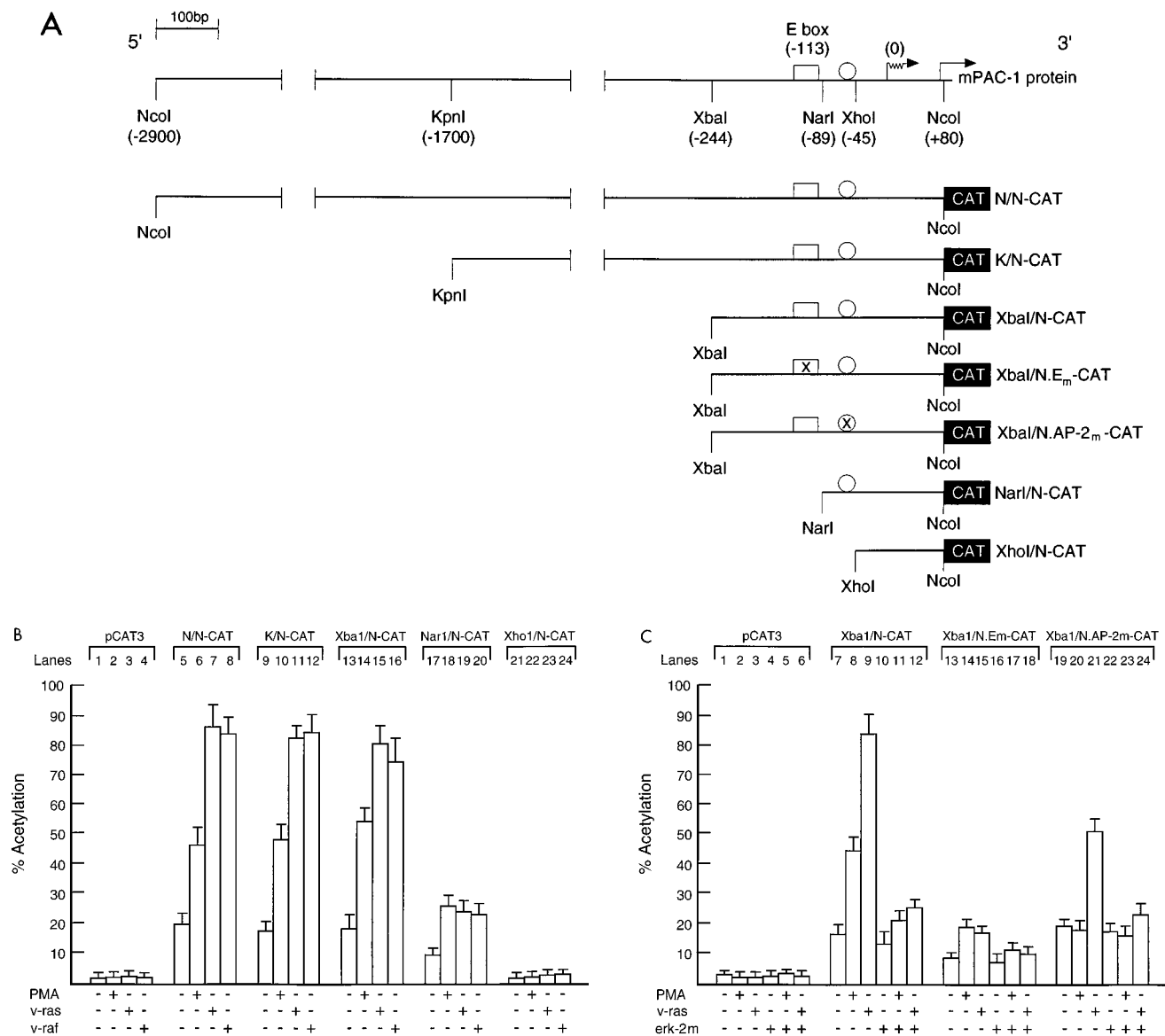


FIG. 2. Functional analysis of the murine *PAC-1* promoter. (A) Schematic diagram of the murine *PAC-1* 5' flanking region and CAT reporter plasmids. The numbers in parentheses indicate the positions of restriction sites in the murine *PAC-1* 5' flanking region according to the numbering outlined by Gerondakis et al. (12). The open box and circle represent the E-box and AP-2-like elements, respectively, while the corresponding symbols with a cross represent the mutated motifs. The wavy arrow denotes the major transcription initiation site, and the straight arrow denotes the murine *PAC-1* initiation codon. The CAT gene is depicted as a closed box. Plasmid nomenclature is indicated to the right of the constructs. (B) PMA, v-Ras, and v-Raf activate murine *PAC-1* transcription in T cells. Jurkat cells carrying stable integrated copies of the *PAC-1* promoter CAT plasmids shown in panel A were generated by cotransfection with 2  $\mu$ g of pSV2Neo and a 10-fold molar excess of each *PAC-1* CAT plasmid as described in Materials and Methods. Pooled independent G418-resistant cells were subsequently stimulated with PMA (lanes 2, 6, 10, 14, 18, and 22) or transiently transfected with 10  $\mu$ g of either the pEFbos expression plasmid containing no insert (lanes 1, 5, 9, 13, 17, and 21), the *v-ras* expression plasmid pEFbos.*v-ras* (lanes 3, 7, 11, 15, 19, and 23), or the *v-raf* expression plasmid pEFbos.*v-raf* (lanes 4, 8, 12, 16, 20, and 24). (C) Mutations in the E-box and AP-2-like motifs reduce PMA and v-Ras-dependent *PAC-1* transcription. The *PAC-1* promoter-CAT plasmid transfectants were transiently transfected with equivalent molar concentrations of pEFbos (lanes 1, 2, 7, 8, 13, 14, 19, and 20), pEFbos.*v-ras* (lanes 3, 6, 9, 12, 15, 18, 21, and 24), or pEFbos.ERK-2<sub>m</sub> (lanes 4, 5, 6, 10, 11, 12, 16, 17, 18, 22, 23, and 24). Approximately 48 h posttransfection, pEFbos (lanes 2, 8, 14, and 20) and pEFbos.ERK-2<sub>m</sub> (lanes 5, 11, 17, and 23) transfectants were treated with PMA for 4 h prior to cell harvesting. The experimental results summarized in panels B and C represent the mean percentage of chloramphenicol acetylation obtained from each of five separate sets of transient transfections.

or NIH 3T3 fibroblasts, both of which had been stably transfected with control plasmids or expression vectors encoding *v-ras* and *v-raf*. The results of these experiments are presented in Fig. 4. Two complexes binding to Ewt, designated Ca and Cb, were detected in both Jurkat (Fig. 4A, lane 1) and NIH 3T3 (12a) nuclear extracts. Although Ca and Cb binding was competed for with an excess of Ewt (lane 4), only Cb binding was competed for with Emut (lane 5). This result together with

the observation that the Ca complex was absent when Emut was used as a probe (lanes 6 and 7) indicates that Ca, but not Cb, specifically bound to the E-box site. Although no novel Ewt-binding complexes were detected in nuclear extracts from cells expressing *v-ras*, Ca but not Cb binding activity increased approximately threefold in these cells (lane 2) but not in Jurkat cells transfected with the nonfunctional *v-ras* G26 mutant (43) (lane 3). This upregulation in Ca binding activity was specific,

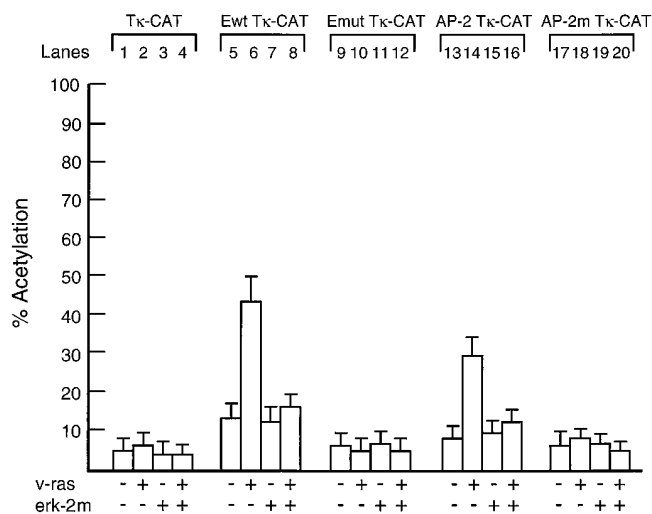


FIG. 3. The E-box and AP-2-like sites confer *v-ras* inducibility upon a heterologous promoter. Jurkat cells were cotransfected with 1  $\mu$ g of the various TK-CAT reporter plasmids and a threefold molar excess of either the pEFbos expression plasmid with no insert (lanes 1, 5, 9, 13, and 17), the *v-ras* expression plasmid pEFbos.*v-ras* (lanes 2, 6, 10, 14, and 18), the mutant ERK-2 expression plasmid pEFbos.ERK-2<sub>m</sub> (lanes 3, 7, 11, 15, and 19), or both the *v-ras* and mutant ERK-2 expression plasmids (lanes 4, 8, 12, 16, and 20). The percentage of acetylated chloramphenicol represents the mean from four independent experiments.

since levels of the Sp1 transcription factor remain unchanged in control and *v-ras*-transfected cells (lanes 1 to 3).

To determine if an increase in Ca binding activity also accompanies induced *PAC-1* transcription in normal cells, nuclear extracts isolated from stimulated splenic B and T cells were subjected to gel shift analysis (Fig. 4B). While there was little detectable Ca binding activity in resting B cells (lane 1), Ca binding was upregulated after a 4-h LPS (lane 2) or anti-CD40 (lane 3) treatment. Similarly, in T cells, both ConA (lane 5) and PMA plus ionomycin (lane 6) stimulation markedly upregulated Ca binding. No increase in Sp1 levels occurred during splenic B- or T-cell activation (12a). Consistent with a possible role for Ca in the induced transcription of *PAC-1* in normal lymphocytes, the upregulation of Ca binding activity coincided with induced *PAC-1* expression in these cells (Fig. 1B and C). Interestingly, in nonstimulated cells, basal Ca levels are higher in T cells than B cells and levels of the Cb complex appear to vary depending on the stimulus.

SDS-polyacrylamide gel electrophoresis analysis of the protein-DNA adducts generated by UV cross-linking Ca to a radiolabeled Ewt probe was used to establish if the induced Ca binding activity isolated from *v-ras*-transfected Jurkat cells and normal stimulated B and T cells was equivalent to that of the basal Ca complex (Fig. 4C). A major adduct of approximately 53 kDa was detected in Jurkat transfectants expressing *v-ras* (lane 2) and the *v-ras* G26 mutant (lane 3), indicating that a protein of the same molecular weight bound to the E box as part of the basal and *v-ras*-induced transcriptional complex. In both resting and activated normal B and T cells (lanes 5 to 10), the major protein-DNA adduct also displayed a molecular mass of 53 kDa. A low-abundance cross-linked molecule of approximately 48 kDa was sometimes present in the Ca complex isolated from Jurkat cells (lanes 2 and 3) and activated splenic B and T cells (lanes 6, 7, 9, and 10), although the ability to detect this species varied between experiments (12a). While a greater abundance of the 53-kDa protein-DNA adduct in stimulated than in resting B and T cells was consistent with an

upregulation of Ca binding in normal activated lymphocytes, the levels of the 53-kDa cross-linked adduct appeared to be equivalent in control and *v-ras*-expressing Jurkat cells, despite a modest threefold increase in *v-ras*-dependent Ca binding.

The protein binding properties of the -89 to -40 region of the *PAC-1* promoter, shown to encompass an element(s) required for PMA and *v-ras*- or *v-raf*-mediated transcriptional transactivation, was also examined by using gel shift analysis. The results are summarized in Fig. 5. A single nuclear complex from Jurkat (C $\beta$  [lane 1]) and NIH 3T3 (results not shown) cells bound to a probe (Inwt) encompassing this region. In contrast, a novel complex (C $\alpha$ ) was detected in addition to the C $\beta$  complex in nuclear extracts from cells expressing *v-ras*

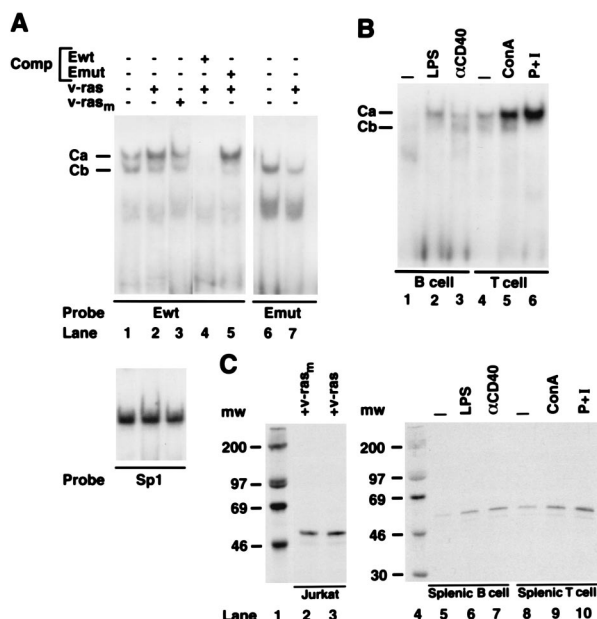


FIG. 4. Properties of complexes that bind to the E-box motif in the murine *PAC-1* promoter. EMSAs were performed as described in Materials and Methods. (A) Identification of a complex that specifically binds to the E box. EMSAs using Ewt (lanes 1 to 5) and Emut (lanes 6 and 7) probes were performed on nuclear extracts derived from Jurkat cells stably transfected with the expression vector pEF-PGK*puro* containing no insert (lanes 1 and 6), *v-Ha-ras* (lanes 2, 4, 5, and 7), or mutant *v-Ha-ras* G26 (lane 3), with no competition (Comp) (lanes 1, 2, 3, 6, and 7) or competition with a 25-molar excess of unlabeled Ewt (lane 4) or Emut (lane 5). Two complexes, designated Ca (upper complex) and Cb (lower complex), are indicated. EMSAs using an Sp1 binding site show that Sp1 levels remain unchanged in the transfected Jurkat cells (lanes 1 to 3). Identical results were obtained with nuclear extracts isolated from NIH 3T3 fibroblasts transfected with this set of expression vectors (data not shown). (B) E-box-binding complexes in normal B and T cells. Splenic B and T cells were purified as described previously (28). Nuclear extracts isolated from unstimulated B cells (lane 1) and T cells (lane 4), B cells treated for 4 h with LPS (lane 2) or anti-CD40 ( $\alpha$ CD40; lane 3), and T cells stimulated for 24 h with ConA (lane 5) or PMA plus ionomycin (P+I; lane 6) were subjected to EMSA using the Ewt probe. Equivalent amounts of protein were used for all binding reactions. Complexes Ca and Cb are indicated. The gel was exposed for 12 h of autoradiography. (C) UV cross-linking analysis of the DNA-binding proteins in the Ca complex. Jurkat T-cell or splenic B- and T-cell nuclear extracts used for experiments shown in Fig. 3A and B, respectively, were subjected to EMSA using a bromo-dUTP-substituted Ewt probe as described in Materials and Methods. After UV irradiation, Ca complexes were eluted and the resultant DNA-protein adducts were analyzed on SDS-10% polyacrylamide gels. Lanes contained <sup>14</sup>C-labeled protein molecular weight (mw) standards (indicated in thousands) (lanes 1 and 4), cross-linked Ca from Jurkat cells transfected with pEF-PGK*puro.v-ras<sub>m</sub>* (lane 2) or pEF-PGK*puro.v-ras* (lane 3), cross-linked Ca from resting B cells (lane 5) and B cells stimulated with LPS (lane 6) or anti-CD40 (lane 7), and cross-linked Ca from resting T cells (lane 8) and T cells stimulated with ConA (lane 9) or PMA plus ionomycin (lane 10). The gels corresponding to lanes 1 to 3 and 4 to 10 were exposed for 18 and 24 h of autoradiography, respectively.

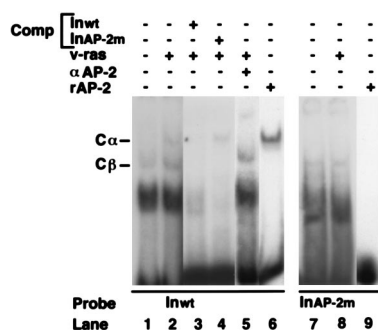


FIG. 5. A *v-ras*-inducible complex binds to the AP-2-like motif in the murine *PAC-1* promoter. EMSAs using Inwt (lanes 1 to 6) and InAP-2<sub>m</sub> (lanes 7 to 9) probes were performed with nuclear extracts isolated from Jurkat cells transfected with pEF-PGKpuro (lanes 1 and 7) or pEF-PGKpuro.v-ras (lanes 2, 3, 4, 5, and 8). Gel shifts using extracts from Jurkat cells transfected with pEF-PGKpuro.v-ras<sub>m</sub> were equivalent to those shown in lanes 1 and 7 (results not shown). Competition (Comp) analysis with a 25-fold molar excess of probe was done with Inwt (lane 3) and InAP-2<sub>m</sub> (lane 4). The upper (C $\alpha$ ) and lower (C $\beta$ ) complexes are indicated. Supershifts with anti-AP-2-specific serum ( $\alpha$ AP-2) were performed on extracts from pEF-PGKpuro.v-ras-expressing Jurkat cells (lane 5), while binding of purified human recombinant AP-2 (rAP-2) to Inwt and InAP-2<sub>m</sub> probes is shown in lanes 6 and 9, respectively.

(lane 2) or *v-raf* (results not shown). The specificity of C $\alpha$  and C $\beta$  binding to Inwt was demonstrated by competition analysis (lane 3). Scrutiny of the nucleotide sequence between -89 to -40 for regulatory elements implicated in the expression of early-response genes revealed the element 5'-GGCCAGGC-3', located at position -52 to -45, that is related to an AP-2 binding motif (38, 55). Consistent with a possible role of this AP-2 site in *v-ras*- or *v-raf*-induced *PAC-1* transcription, AP-2 binding has been shown to be induced by multiple stimuli, including phorbol ester and those that activate MAP kinase (22, 25, 32). Binding studies using a probe spanning -89 to -40, in which the putative AP-2 site had been mutated (InAP-2mut), detected only complex C $\beta$  in extracts from Jurkat cells expressing *v-ras* (lane 8). Competition analysis with InAP-2mut, which resulted in the loss of C $\beta$  but not C $\alpha$  binding (lane 4), further established the specificity with which C $\alpha$  bound to the putative AP-2 motif. Two independent lines of evidence were used to establish that the C $\alpha$  complex was AP-2. First, antibodies specific for AP-2 supershifted the C $\alpha$  but not the C $\beta$  complex (lane 5). Second, recombinant human AP-2 bound to Inwt (lane 6) with a mobility equivalent that of C $\alpha$  but failed to bind the InAP-2mut probe (lane 9).

Plasmid XbaI/N-CAT, in which the AP-2 site was specifically mutated such that C $\alpha$  no longer bound, was transfected into approximately two- to threefold by *v-ras* (Fig. 2C, lane 21). This supports results of the binding studies that link the *v-ras*- or *v-raf*-activated C $\alpha$  complex to inducible *PAC-1* transcriptional transactivation. Moreover, the inability of PMA to up-regulate XbaI/N.AP-2<sub>m</sub>-CAT (lane 20) indicated that phorbol ester-mediated *PAC-1* transcription also operates through the AP-2 site. Consistent with a role for the AP-2 element in *v-ras*-induced *PAC-1* transcription, the wild-type (Fig. 3, lane 14) but not the mutant (lane 18) AP-2 motif was able to confer a *v-ras*-dependent transcriptional induction upon the TK promoter-CAT reporter plasmid. Both the PMA- and the *v-ras*-mediated AP-2-dependent increase in *PAC-1* transcription appeared to require MAP kinase function, since the interfering ERK-2 mutant ablated transactivation of the *PAC-1* promoter reporter (Fig. 2C, lanes 17 and 18) and AP-2TK-CAT (Fig. 3, lane 16) plasmids. Together, these studies establish that MAP

kinase-dependent *PAC-1* transcription in T cells is mediated by AP-2 and an E-box-binding factor.

## DISCUSSION

While much is known about how the activation of different MAP kinases by phosphorylation occurs in response to a variety of extracellular signals, little is understood about the role that phosphatases play in the downregulation of MAP kinase function. Here we show that in hemopoietic cells, the expression of *PAC-1* MAP kinase phosphatase is modulated by stimuli that induce proliferation and differentiation and that *PAC-1* transcription in T cells is upregulated by engagement of the MAP kinase signaling pathway.

Expression studies revealed that there are differences in the pattern of *PAC-1* expression between B and T lymphocytes. In the B-cell lineage, although *PAC-1* mRNA is expressed constitutively in tumors representative of surface IgM-positive B lymphocytes, it is undetectable in resting splenic B cells prior to mitogenic stimulation. While the basis of the difference between normal B cells and cell lines is unclear, constitutive expression is unlikely to simply reflect the proliferative status of immortalized B cells, since *PAC-1* is not detected in plasmacytomas. During normal B-cell mitogenesis, a similar pattern of induced *PAC-1* expression occurs in response to different stimuli. Expression is rapidly induced but is downregulated by 24 h. This contrasts with primary T cells, in which induced expression is sustained for up to 24 h, with the kinetics of maximal expression depending on the type of stimulus. Although it remains to be determined which activated MAP kinases in addition to ERK-2 (50, 51) and p38 (45) are *PAC-1* substrates, if *PAC-1* is induced directly in response to increased MAP kinase activity, then elevated levels of *PAC-1* mRNA in hemopoietic cells may be an indirect indicator of MAP kinase activation. If true, this would indicate that in B cells, MAP kinase activity regulated by *PAC-1* appears to be the same regardless of the mode of activation, whereas in T cells, MAP kinase activity varies depending on the type of mitogenic stimulus.

While induced *PAC-1* expression in lymphocytes is associated with mitogenesis, during hemopoietic cell differentiation, changes in *PAC-1* expression do not appear to be intimately linked to cellular proliferation. In the M1 myeloid cell line, *PAC-1* expression is rapidly downregulated by differentiation-inducing agents but is upregulated again by day 6 when differentiation into a nondividing macrophage population is complete. In contrast, *PAC-1* expression is switched off in the proliferating macrophage/granulocyte population that arises from IL-3 treatment of SPGM1 pre-B cells. Collectively, the different patterns of *PAC-1* expression indicate that *PAC-1* is likely to be involved in the regulation of a number of MAP kinase-dependent physiological processes that occur during the proliferation and differentiation of hemopoietic cells.

*PAC-1* transcription induced in T cells is mediated through two discrete elements in the promoter: an E-box motif that is also required for basal transcription and an AP-2-like binding site. The modest two- to threefold PMA-dependent induction of *PAC-1* transcription in Jurkat cells requires the AP-2-like site but not the E-box motif. By contrast, maximal *v-ras*- or *v-raf*-mediated transcriptional transactivation is dependent on both sites, with the mutation of either site alone reducing *v-ras*- or *v-raf*-dependent transactivation and the loss of both sites completely ablating transcription.

The signaling pathway(s) proposed to be responsible for induced *PAC-1* expression is outlined schematically in Fig. 6. The implied involvement of MAP kinase is reinforced by the

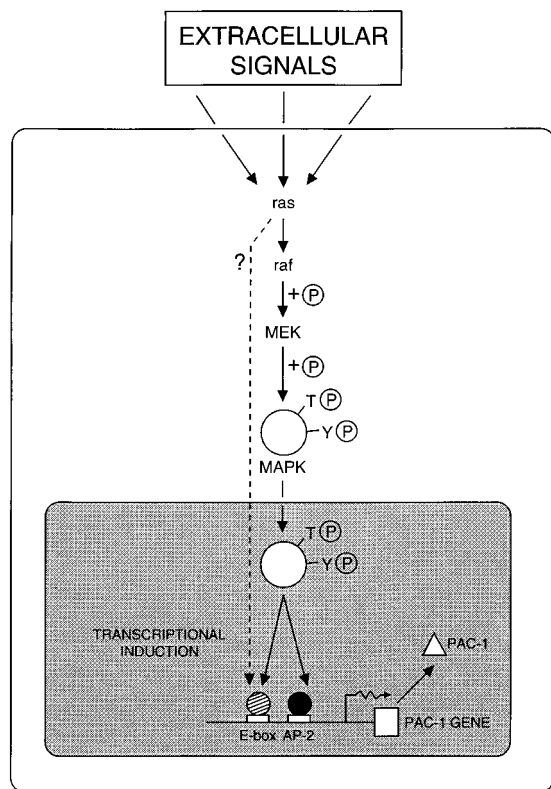


FIG. 6. Activation of the MAP kinase (MAPK) signal transduction pathway induces *PAC-1* transcription in T cells. The stippled and closed circles represent the 53-kDa E-box DNA-binding protein and AP-2, respectively. The broken line corresponds to an unidentified signaling pathway that may be involved in E-box-dependent *PAC-1* transcription.

finding that a constitutively activated mutant of MEK (8) induces *PAC-1* transcription in T cells that is dependent on the E-box and AP-2 elements (12a) and that an interfering ERK-2 mutant blocks *v-ras*-dependent transactivation. While the data presented here indicate that MAP kinase is required for *PAC-1* transcription, it remains unclear why PMA is unable to induce maximal transactivation of *PAC-1* promoter-reporter plasmids, despite phorbol esters having been shown to activate MAP kinase (7, 46). This could possibly be due to differences in the degree or duration of MAP kinase activation by PMA stimulation and *v-ras* expression, or possibly *v-ras* and *v-raf* also activate a MAP kinase-independent pathway(s). If another signaling pathway is involved in the induction of *PAC-1* transcription, then transfection analysis of the promoter constructs indicates that it would have to regulate transcription via the E-box. This would mean that MAP kinase activation is necessary but not sufficient for the E-box-dependent component of induced *PAC-1* transcription. Consistent with such a model of *PAC-1* transcription are the findings that both *ras* and *raf* activate other signaling pathways. *ras* can activate a phosphatidylinositol-3-OH kinase-dependent pathway (47), while in Rat-1 fibroblasts, *v-raf* activates N-terminal Jun kinase (36). It remains to be determined if all stimuli that induce *PAC-1* transcription require the MAP kinase pathway, what other pathways are involved in regulating *PAC-1* transcription, and whether novel sequences in the *PAC-1* promoter are involved in extracellular signal-dependent transcription.

The involvement of AP-2 in induced but not basal *PAC-1* transcription is consistent with findings that show AP-2 DNA

binding activity is upregulated by PMA and second messengers that activate MAP kinase (22, 23). The regulation of Ca binding activity appears to be complex. Although binding of Ca to the E box is intimately linked with basal *PAC-1* transcription in Jurkat cells, increased Ca binding activity is also associated with *v-ras*- or *v-raf*-dependent transactivation of *PAC-1* promoter reporter plasmids. In normal B and T cells, Ca binding is upregulated in response to stimuli that induce *PAC-1* transcription. The inability to detect *PAC-1* expression in normal resting lymphocytes, despite the presence of the Ca complex, indicates that in these cells Ca alone is insufficient to induce *PAC-1* expression.

While the evidence from transfection studies suggests that the *v-ras*-mediated E-box dependent transactivation involves MAP kinase and possibly an unidentified signaling pathway, it remains to be determined precisely how such pathways regulate this transcriptional response. In Jurkat cells, the 53-kDa DNA-protein adduct is the major DNA binding activity detectable in the Ca complex isolated from unstimulated or *v-ras*-transfected Jurkat cells. Similarly, in normal lymphocytes, the 53-kDa cross-linked species was present in the Ca complex. Occasionally a 48-kDa DNA-protein adduct of varying abundance was also detected in Ca isolated from unstimulated and activated Jurkat cells and splenic lymphocytes. Preliminary evidence suggests that it may be a proteolytic breakdown product of the 53-kDa adduct (12a). Despite an increase in Ca binding activity induced by *v-ras*, no change was observed in the abundance of the 53-kDa DNA binding protein in Jurkat cells. Although it can be difficult to detect small changes in the abundance of DNA-binding proteins in cross-linking analysis, it was possible to detect increased levels of the 53-kDa protein in the Ca complex from normal stimulated B and T cells (Fig. 4C). These differences may indicate that Ca binding is regulated differently in normal lymphocytes and Jurkat cells. For example, in addition to increased levels of the 53-kDa protein, enhanced Ca DNA binding activity could result from a post-translational modification or a non-DNA-binding component of the Ca complex increasing the DNA binding affinity of the 53-kDa protein. The latter model is consistent with the phosphorylation of transcription factors such as Elk-1 (20, 51), c-Jun (11), and AFT-2 (18) by MAP kinases increasing the transcriptional activity of these proteins. Purification and characterization of the 53-kDa protein should provide insights into the mechanism(s) by which the MAP kinase and other signaling pathways regulate the activity of this transcription factor.

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