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Nuclear Localization of v-Abl Leads to Complex Formation with Cyclic AMP Response Element (CRE)-Binding Protein and Transactivation through CRE Motifs

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Deregulated expression of v-*abl* and *BCR/abl* genes has been associated with myeloproliferative syndromes and myelodysplasia, both of which can progress to acute leukemia. These studies identify the localization of the oncogenic form of the *abl* gene product encoded by the Abelson murine leukemia virus in the nuclei of myeloid cells and the association of the v-Abl protein with the transcriptional regulator cyclic AMP response element-binding protein (CREB). We have mapped the specific domains within each of the proteins responsible for this interaction. We have shown that complex formation is a prerequisite for transcriptional potentiation of CREB. Transient overexpression of the homologous cellular protein c-Abl also results in the activation of promoters containing an intact CRE. These observations identify a novel function for v-Abl, that of a transcriptional activator that physically interacts with a transcription factor.

Transformation by v-Abl, a nonreceptor tyrosine kinase, results in uncontrolled tyrosine phosphorylation of substrates that signal the regulation of cell growth and transformation (6, 27). Abelson leukemia virus is an acute transforming retrovirus of murine origin (1). The p160 v-Abl gene product of Abelson murine leukemia virus arose by a recombination event in which sequences encoding the amino-terminal region of c-Abl, including *src* homology region 3 (SH3), were substituted by *gag* encoding sequences of Moloney murine leukemia virus (6, 54). Aside from the N-terminal truncation (fusion), the major difference between c-Abl and v-Abl has been identified as a 23-amino-acid frameshift downstream from the tyrosine kinase domain, within a region not required for its transforming activity (26). The p120 strain used in the present study was derived from p160 v-Abl by an in-frame deletion of 789 internal nucleotides and is an oncogenic protein with tyrosine kinase activity (37, 48, 49).

In myeloid cells, growth factors activate protein tyrosine kinases to transmit their mitogenic signals (18), and deregulated, constitutive expression of tyrosine kinases has been shown to cause neoplastic transformation (35). Evidence from previous studies supports v-Abl's ability to affect the mitogenic pathway by induction of c-*myc* expression (8). In addition, the cellular homolog c-Abl has been shown to interact with the nuclear retinoblastoma gene product through its kinase domain (56) and to bind nonspecifically to DNA via its carboxy-terminal domain (25). Both the kinase and the DNA binding carboxy-terminal domains are present in p120 v-Abl (30, 39) (see Fig. 8).

One of the best-characterized families of transcription factors recognizes a consensus DNA sequence known as the CRE (cyclic AMP [cAMP] response element). CREs are known to mediate transcriptional responses resulting from an increase in intracellular cAMP levels (41). The 43-kDa CRE-binding protein (CREB), which binds the consensus motif 5′-TGACGT CA-3′ and activates transcription, was initially isolated from rat brain tissue (31, 57) and found to be ubiquitously expressed (5, 12). CREB belongs to the leucine zipper class of proteins, of which at least nine members are known to date (5, 29). Certain leucine zipper family members can form heterodimers in specific combinations; however, the functional significance of this specific pairing is unknown. The carboxy terminus of CREB contains a leucine zipper that is required for dimerization and DNA binding (53). CREB also has a transactivation domain which contains several independent regions, including one identified as the kinase-inducible domain (KID). The KID contains consensus phosphorylation sites for different kinases, including protein kinase A (5). One of the known mechanisms of CREB transcriptional activation is phosphorylation of Ser-133 by the catalytic subunit of cAMP-activated protein kinase A (41). It has been suggested that phosphorylation events alter the conformation of the transactivation domain of CREB, causing enhanced interaction with the transcriptional machinery (17).

Using murine myeloid cell lines, we have explored the biochemical pathways that signal the regulation of gene expression by v-Abl. An interleukin-3 (IL-3)-dependent cell line (32D-123 [15]) was infected with Moloney murine leukemia virus containing v-*abl*, yielding the growth factor-independent 32D-abl cell line (23). Our current studies demonstrate the ability of these cells to transactivate chimeric promoter-chloramphenicol acetyltransferase (CAT) constructions containing CRE motifs. Moreover, we have shown that the p120 v-Abl gene product localizes to the nucleus and cytoplasm of 32D-abl myeloid cells and have identified an independently functioning nuclear translocation signal within the v-Abl protein. These studies also demonstrated a physical association between v-Abl

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and the CREB transcription factor involving a region of CREB (amino acids 150 to 260), which includes a glutamine-rich domain, and a region of the v-Abl protein which consists of the SH2 domain and the ATP binding region. The association between CREB and v-Abl identifies a new protein-protein transactivation function for v-Abl.

MATERIALS AND METHODS

Cell lines. 32D-123 cells were grown in RPMI 1640, in the presence of 10% fetal bovine serum and 10 ng of recombinant murine IL-3 per ml or 10% Walter and Elisa Hall Institute conditioned media. 32D-abl cells were established by infecting the 32D-123 cell line with Moloney murine leukemia virus containing the v-*abl* gene as previously reported (23). Human mononuclear cells, from normal donors who had previously given their consent, were obtained by Ficoll-Hypaque gradient centrifugation. T cells were separated from monocytes by countercurrent centrifugal elutriation, purified in a human T-cell column (R and D Systems, Valencia, Calif.), and then resuspended in RPMI 1640 (Gibco BRL, Grand Island, N.Y.) containing 10% fetal bovine serum (HyClone, Logan, Utah) in the presence of phytohemagglutinin (1 mg/ml; Sigma, St. Louis, Mo.). After 3 days in culture, the cells were used as indicated below.

Plasmids. The following transforming growth factor β3 (TGF-β3) promoter-CAT constructions (including deletion mutants) were described previously (28). Briefly, the series of TGF-β3–CAT chimeric plasmids were constructed starting 110 nucleotides 3′ of the TGF-β3 transcription start site (+110) and extending 5′ to the number indicated in the plasmid name. pB3-499 contains the TATA, CRE, and AP-2 consensus sequences, and pB3-499ΔCRE contains a two-nucleotide mutation in the CRE (28). Additional chimeric promoter-CAT plasmids were constructed by PCR amplification and ligation of the CRE sites from a variety of heterologous promoters upstream of the minimal human TGF-β2 promoter (−68 to +63) contained in pGEM4-SVOCAT. These constructions were verified first by restriction enzyme analysis and then by DNA sequence analysis. The control plasmid pB2-40, which does not contain a CRE sequence, was also generated by PCR with pB2-68 as a template. The v-*abl* expression vector used in these studies encodes the p120 protein and has been described previously (13, 46, 49), as has the human T-cell leukemia virus type 1 CAT plasmid (20). The c-*abl* expression vector was a generous gift from Ann Marie Pendergast (Duke University Medical Center, Durham, N.C. [34]).

The GAL4-CREB, GAL4-CREBΔLZ, and GAL4-CREBΔLZM1 constructions used in these experiments were a generous gift from Margaret A. Thomson and were previously described (45). GAL4-CREB contains the complete 341 amino acids of CREB fused to the DNA binding domain of GAL4 (amino acids 1 to 147). GAL4-CREBΔLZ lacks the COOH-terminal 29 amino acids of GAL4-CREB, including the leucine repeat dimerization motif; GAL4-CREBΔLZM1 contains a change from Ser-133 to Ala in GAL4-CREBΔLZ. pSG-147 is the DNA binding domain of GAL4 (24). The reporter construction G5B contains five GAL4 binding sites upstream of the adenovirus E1b TATA box driving CAT (24).

For in vitro transcription and translation in rabbit reticulocyte extracts, various portions of the v-*abl* coding regions were placed in frame behind the phage T7 promoter in the pGEM4 plasmid (Promega, Madison, Wis.). v-*abl* DNA fragments were produced by PCR. The 5′ oligonucleotide used in all amplifications contained a *Bam*HI site, and the 3′ oligonucleotide contained an *Eco*RI site. With these oligonucleotides, fragments were amplified according to the standard protocol of the GeneAmp kit (Perkin-Elmer, Norwalk, Conn.). The junctions of all plasmids were confirmed by DNA sequencing.

GST fusion constructions and reactions. Using oligonucleotides specific to the different domains of the CREB gene, we applied PCR amplification to clone various regions of the CREB coding sequence into the pGST-2T expression vector (Pharmacia, Piscataway, N.J.) as *Bam*HI-*Eco*RI fragments, thus generating different glutathione *S*-transferase (GST)-C1, -C2, -C3, -C4, -C5, and -C6 constructions (see Fig. 7). CREB affinity matrices were constructed by using GST-CREB fusion proteins expressed in *Escherichia coli* as described previously (21). The affinity matrices were prewashed in elution buffer, subsequently equilibrated in NETN buffer (see GST-pull-down assay protocol below), and incubated with total cellular extracts from either 32D-123 or 32D-abl cells in NETN buffer. Bound proteins were detected by immunoblotting with antibodies specific to Abl.

CAT assays. The murine myeloid 32D-123 and 32D-abl cells were transfected by the electroporation method, as previously described (4). Equal concentrations of DNA were transfected in each case by the addition of pUC18 when necessary. After 24 h at 37°C the cells were harvested, and the protein concentrations of the cell lysates were determined by using the Bio-Rad (Richmond, Calif.) protein assay. CAT enzyme activity was normalized for transfection efficiency by cotransfection with the human growth hormone expression plasmid pXGH5, and the amount of secreted growth hormone in the medium was determined prior to harvesting for CAT activity by radioimmunoassay (Nichols Institute, San Juan Capistrano, Calif.). Equal amounts of protein were used to assay for the CAT enzyme, and each assay was repeated at least three times. Quantification of the percent acetylation was done with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Immunoprecipitations and Western blot (immunoblot) analysis. Approximately 1×10^7 to 2×10^7 cells were prepared by lysis with 100 μl of RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris [pH 8.0]), containing phosphatase inhibitors when the cells were ^{32}P labelled (1 mM sodium orthovanadate, 30 mM NaF, 30 mM $NaPP_i$) and 1% bovine serum albumin (BSA). Antibody (1 μg) was incubated with the extracts for 1 h at 4°C. The antigen-antibody complexes were precipitated with protein G-Sepharose Plus (Santa Cruz Biotechnology, Santa Cruz, Calif.), washed three times with RIPA buffer, and then separated by SDS-polyacrylamide gel electrophoresis (PAGE). The separated proteins were transferred to reinforced nitrocellulose membranes (Schleicher and Schuell, Keene, N.H.) and probed by Western blot analysis. The antigen-antibody complexes were detected by enhanced chemiluminescence by following the manufacturer's instructions (Amersham, Arlington Heights, Ill.).

In vivo phosphorylation was carried out by labelling the 32D cells with inorganic ^{32}P for 2 h at 37°C. ^{32}P-labelled cell extracts were immunoprecipitated with specific antisera as indicated in the figures and then analyzed by SDS-PAGE and autoradiography.

Nuclear and cytoplasmic lysates. Cells (10^7) were pelleted and washed with cold phosphate-buffered saline (PBS). The pellets were resuspended in 200 μl of buffer A (0.005 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] [pH 8.0], 0.0015 M $MgCl_2$, 0.010 M KCl), immediately pelleted, and resuspended in 200 μl of buffer A. After a 10-min incubation on ice, the cytoplasmic fraction was separated by centrifugation at 3,000 rpm in an Eppendorf centrifuge. Next, the pellet was resuspended in an equal volume of buffer B (0.02 M HEPES [pH 8.0], 25% glycerol, 0.5 M NaCl, 0.0015 M $MgCl_2$, 0.0005 M EDTA] and incubated on ice for 30 min. The suspension was centrifuged at 70,000 rpm in a TLA 100 (Beckman) rotor for 45 min at 4°C, and the supernatant containing the nuclear fraction was stored at −70°C.

Antibodies. A c-Abl (24-11) mouse monoclonal immunoglobulin G antibody corresponding to the carboxy-terminal domain of v-Abl, which is known to react with both v- and murine c-*abl*-encoded proteins, was used in the Western blot and immunoprecipitation studies. An anti-CREB (240) rabbit polyclonal antibody and the peptide that it was raised against were used in the immunoprecipitation and Western blot studies. An affinity-purified rabbit polyclonal c-Abl antibody (K-12) raised against an epitope next to the tyrosine kinase domain and its corresponding peptide were used in immunoprecipitation and Western blot studies. All the above-mentioned antibodies were obtained from Santa Cruz Biotechnology, Inc., unless otherwise specified.

GST-pull-down assay. The GST-pull-down assay was performed as previously described (16), with minor modifications. The concentration of GST fusion proteins was determined by Coomassie blue staining. The beads were washed twice with NETN buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) and once with binding buffer (50 mM Tris-HCl [pH 8.0], 140 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 μM sodium orthovanadate, 50 mM Tris-HCl [pH 8.0], 1% BSA). The beads were rocked for 1 h at room temperature with 2 to 10 μl of in vitro-synthesized ^{35}S-v-Abl protein in a final volume of 200 μl of binding buffer. The beads were then washed three times in 1 ml of NETN buffer, pelleted at 500 × *g* for 2 min, and boiled in SDS-PAGE sample buffer; the bound ^{35}S-labelled v-Abl was resolved by SDS-PAGE.

Immunofluorescence and nuclear localization assays. The murine myeloid 32D-123 cells were transfected with the Abl-FL and Abl-M6 constructions as previously described (4). Equal concentrations of DNA were transfected in each case by the addition of pUC18 when necessary. Cytospins of actively growing 32D-123 and 32D-abl cells (1.25×10^5 cells) were prepared on microscope slides and fixed for 5 min with cold methanol-acetone (1:1 mixture, −20°C), rinsed in PBS, and blocked for 1 h in 10% normal goat serum in PBS. The slides were probed with 1 μg of affinity-purified rabbit antiserum to Abl per ml in 5% normal goat serum–0.2% Tween 20 in PBS overnight. The slides were extensively rinsed in 0.2% Tween 20–PBS and probed with a 1:5,000 dilution of biotinylated goat anti-rabbit antibody (Kirkegaard and Perry) for 1 h. After being rinsed in PBS-Tween 20, the slides were probed with a 1:100,000 dilution of fluorescein isothiocyanate-labelled Ultravidin (Leinco Technologies) in PBS–5% normal goat serum–0.2% Tween 20 for 1 h. After being rinsed in PBS-Tween 20, the slides were stained with 1 μg of 4′,6-diamidino-2-phenylindole (DAPI) (DNA staining) in PBS for 5 min and rinsed, and coverslips were applied.

To test putative nuclear localization signals (NLS), we used a system derived from the herpes simplex virus type 1 immediate-early IE175 protein (40). The wild-type IE175 expression vector, pGH114, encodes an exclusively nuclear protein that contains an epitope recognized by monoclonal antibody 58S in the C-terminal region (47). A variant form of the IE175 protein, encoded by expression vector pGH115, contains an in-frame deletion of codons 383 to 833 (including the NLS), resulting in an exclusively cytoplasmic form of this protein. This construction also contains a *Bgl*II linker at the site of the internal deletion. Double-stranded synthetic oligonucleotides encoding two putative NLS for the v-*abl* gene, NLS-1 (5′-GATCTCCCCCAAGCGCAACAAGCCCGG-3′ and 5′-GATCCCGGGCTTGTTGCGCTTGGGGGA-3′) and NLS-2 (5′-GATCTCCC CCAAACTCCTCCGCAGGGG-3′ and 5′-GATCCCCCTGCGGAGGAGTT TGGGGGA-3′), were annealed and cloned in frame into the *Bgl*II site of pGH115. The amino acids encoded by the oligonucleotides were [S]PKRNK-

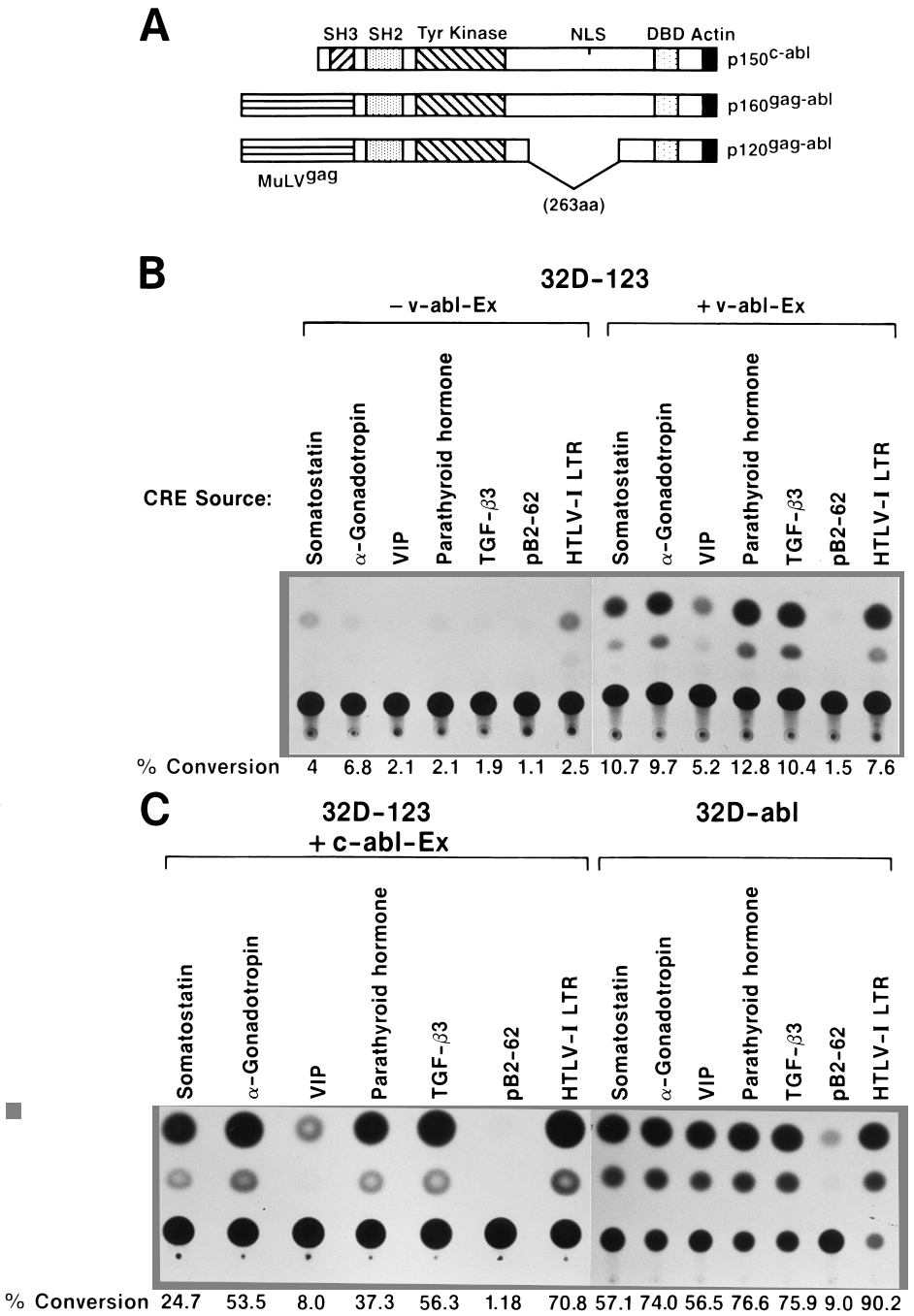

FIG. 1. (A) Diagrammatic comparison of the p150 c-Abl, p160 gag-Abl, and p120 gag-Abl proteins. Characterized regions within each of the three Abelson proteins are indicated as follows: SH3, SH2, tyrosine kinase domain including the ATP binding region (Tyr kinase), NLS, DNA binding domain (DBD), actin binding domain (Actin), and MuLV-derived Gag ($MuLV^{gag}$). aa, amino acids. (B) Chimeric CAT constructions containing CREs from different genes are highly expressed in v-*abl*-transformed myeloid cells. 32D-123 cells (grown in the absence of IL-3) were transiently transfected with 10 μg of a chimeric promoter-CAT construct containing the minimal human TGF-β2 promoter (−68 to +63) and an upstream CRE (three CREs in the case of the human T-cell leukemia virus type 1 long terminal repeat [HTLVI LTR]) from a heterologous gene as shown. Plasmid pB-62 corresponds to the minimal TGF-β2 promoter without an upstream CRE. 32D-123 cells were cotransfected with 15 μg of the v-*abl* expression vector (+v-abl-Ex) or pUC18 (−v-abl-Ex). (C) Expression of CRE-containing promoters in 32D-123 and 32D-abl cells. 32D-123 cells were cotransfected with 15 μg of the c-*abl* expression vector (+c-abl-Ex) and 10 μg of reporter plasmid, while 32D-abl cells were transiently transfected with 10 μg of reported plasmid alone and 15 μg of pUC18. CAT activity was measured 24 h later, and values were normalized as described in Materials and Methods.

P[GI] and [S]PKLLRR[GI] (bracketed residues arose for the restriction sites used in the cloning).

NIH 3T3 cells grown on four-well microscope slide chambers (Lab-Tek; Nunc, Naperville, Ill.) were transfected with 1.5 μg of each test plasmid by the PBS-calcium phosphate technique (7). After 48 h, the slides were washed in PBS and fixed in methanol at −20°C for 15 min. The slides were then air dried, rehydrated with PBS, and incubated with a monoclonal antibody (1:100 dilution in PBS) for 30 min at 37°C in a humidified chamber. The slides were then washed in PBS for 5 min and incubated with a goat anti-mouse immunoglobulin G antibody fraction conjugated to fluorescein isothiocyanate (1:100 dilution in PBS; Cappel) for 30 min at 37°C. Finally, the slides were washed in PBS again for 5 min, overlaid with PBS-glycerol (1:1), and sealed with a coverslip by using nail polish.

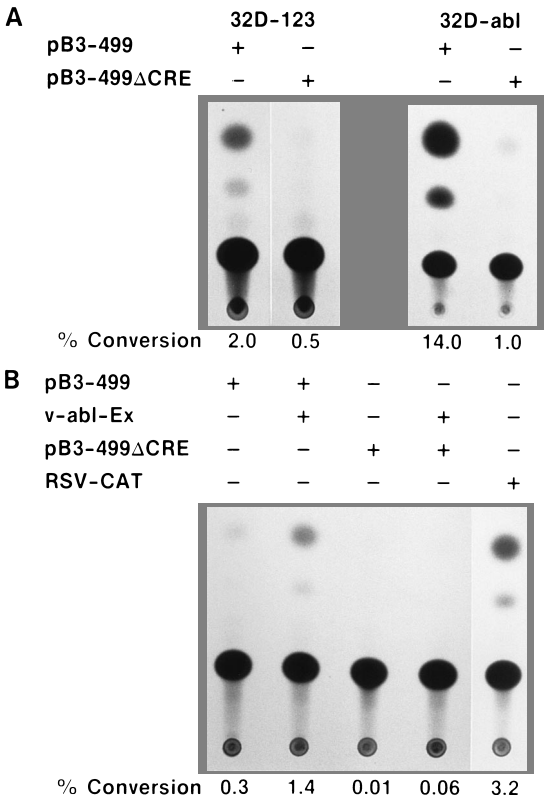

FIG. 2. An intact CRE is critical for induction of TGF-β3 promoter activity by v-Abl. (A) 32D-123 cells (grown in the presence of IL-3) and 32D-abl cells were transfected with 10 μg of wild-type (pB3-499) or mutant (pB3-499ΔCRE) TGF-β3 promoter–CAT constructions. (B) A CRE-containing promoter is activated by v-Abl in T cells. Normal human T cells grown in the presence of PHA were cotransfected with 10 μg of pB3-499 or pB3-499ΔCRE in the presence of 15 μg of pUC18 or the v-*abl* expression vector (v-abl-Ex). Rous sarcoma virus (RSV)-CAT was used as a control. CAT activity was measured 24 h later, and values were normalized as described in Materials and Methods. Results are representative of three independent experiments.

RESULTS

Transcriptional expression of mitogen-responsive genes in v-*abl*-transformed myeloid cells. Sequences for the parathyroid hormone-CRE (55), α-gonadotropin-CRE (10), vasoactive intestinal peptide-CRE (51), somatostatin-CRE (32), and pB3 (TGF-β3) CRE (28) were individually ligated to the human TGF-β2 promoter. Sequence ligation occurred between nucleotides −68 and +63, a region of the TGF-β2 promoter that contains the TATA sequence but lacks the CRE of the TGF-β2 promoter. All plasmids were linked to the CAT gene and were transiently expressed and analyzed for their activities in 32D cells (Fig. 1). High levels of CAT activity were observed for all constructions containing a CRE motif. When the same constructions were transfected into 32D-123 cells grown in the absence of IL-3, very low levels of CAT activity were detected. Because we suspected that IL-3 signals in part through the cAMP pathway, we performed transfection experiments involving Abl activation of CREB in the absence of this cytokine, unless otherwise noted. Cotransfection of these promoters into 32D-123 cells in the presence of exogenous plasmid expressing v-Abl gave high levels of CAT activity resembling those obtained with 32D-abl cells (Fig. 1). Similarly, transient overexpression of cellular c-Abl protein also resulted in similar levels of CAT activity in the 32D-123 cells. These studies indicate that both the transforming p120 v-Abl protein and the nontransforming c-Abl protein can activate genes containing an intact CRE(s), in their promoter, to high levels of expression.

Transactivation requires an intact CRE motif. TGF-β3 is known to be a critical regulator of hematopoietic cell growth, having the most potent antiproliferative (19) capacity among the different members of the TGF-β family. Previous studies have characterized the 5′ flanking region of the TGF-β3 gene and indicated that a CRE is an essential element for the basal transcriptional activity of the promoter (28). Therefore, we chose to use the TGF-β3 gene as a target for the investigation of the mechanism by which v-Abl regulates the transcriptional activity of genes containing CRE in their promoters.

We first analyzed the importance of the CRE consensus sequence in TGF-β3 basal expression in the 32D cell lines. The chimeric TGF-β3 promoter–CAT reporter constructions pB3-499 and pB3-499ΔCRE (a mutation of two sites in the CRE) (28) were transiently expressed in 32D-123 and 32D-abl cells (Fig. 2A). Our results showed that the level of pB3-499 promoter expression was sevenfold higher in 32D-abl cells than in 32D-123 cells grown in the presence of IL-3. Expression of the pB3-499ΔCRE promoter was greatly decreased in 32D-abl cells and was virtually abolished in 32D-123 cells. These results indicate that in myeloid cells, the CRE is critical for the expression of the TGF-β3 promoter, and this motif can be activated by v-Abl.

We also examined the regulation of TGF-β3 expression by v-Abl in human phytohemagglutinin-activated primary T cells. When the primary T cells were cotransfected with pB3-499 and the v-*abl* expression vector, higher levels of activity were observed than in cells cotransfected with pB3-499 and carrier DNA (Fig. 2B). These results demonstrate that v-Abl increases TGF-β3 promoter activity in other primary human T cells and suggest that v-Abl signalling of CREB is not limited to murine myeloid cells.

v-Abl signals transactivation via GAL4-CREB fusions in 32D cells. To investigate the transactivation of CREB without interference from other endogenous CREB family members with similar DNA binding specificities, we used a series of GAL4-CREB fusion activators that contained the DNA binding domain of the *Saccharomyces cerevisiae* transactivator GAL4 fused to either (i) full-length CREB (341 amino acids; GAL4-CREB), (ii) a deletion mutant of CREB lacking the leucine zipper domain (312 amino acids, GAL4-CREBΔLZ), or (iii) a deletion mutant of CREB lacking the leucine zipper motif and including a mutation of Ser-133 to Ala (GAL4-CREBΔLZM1). These constructions were transiently transfected together with a reporter construction containing five GAL4 binding sites upstream of the E1B TATA box driving the CAT gene (24) into 32D-123 and 32D-abl cells (Fig. 3A). Activation of the GAL4-CREB construction occurred in 32D-abl cells, but not in 32D-123 cells, and required both the CREB moiety of the GAL4-CREB fusion protein and the presence of the GAL4 binding sites in the promoter of the reporter gene (Fig. 3B). Moreover, similar levels of activation were observed in 32D-123 cells that had been cotransfected with the p120 v-Abl expression vector.

We next tested the possibility that the GAL4-CREB leucine zipper dimerization motif might interact with an endogenous cell protein(s) that represents the true target of v-Abl activation. When 32D-123 cells were cotransfected with the mutant GAL4-CREB fusion construction lacking the leucine zipper motif (GAL4-CREBΔLZ) and the v-*abl* expression vector, activation of the reporter gene was still observed, demonstrating that the N-terminal 312 amino acids of CREB is the target of activation by v-Abl (Fig. 3C).

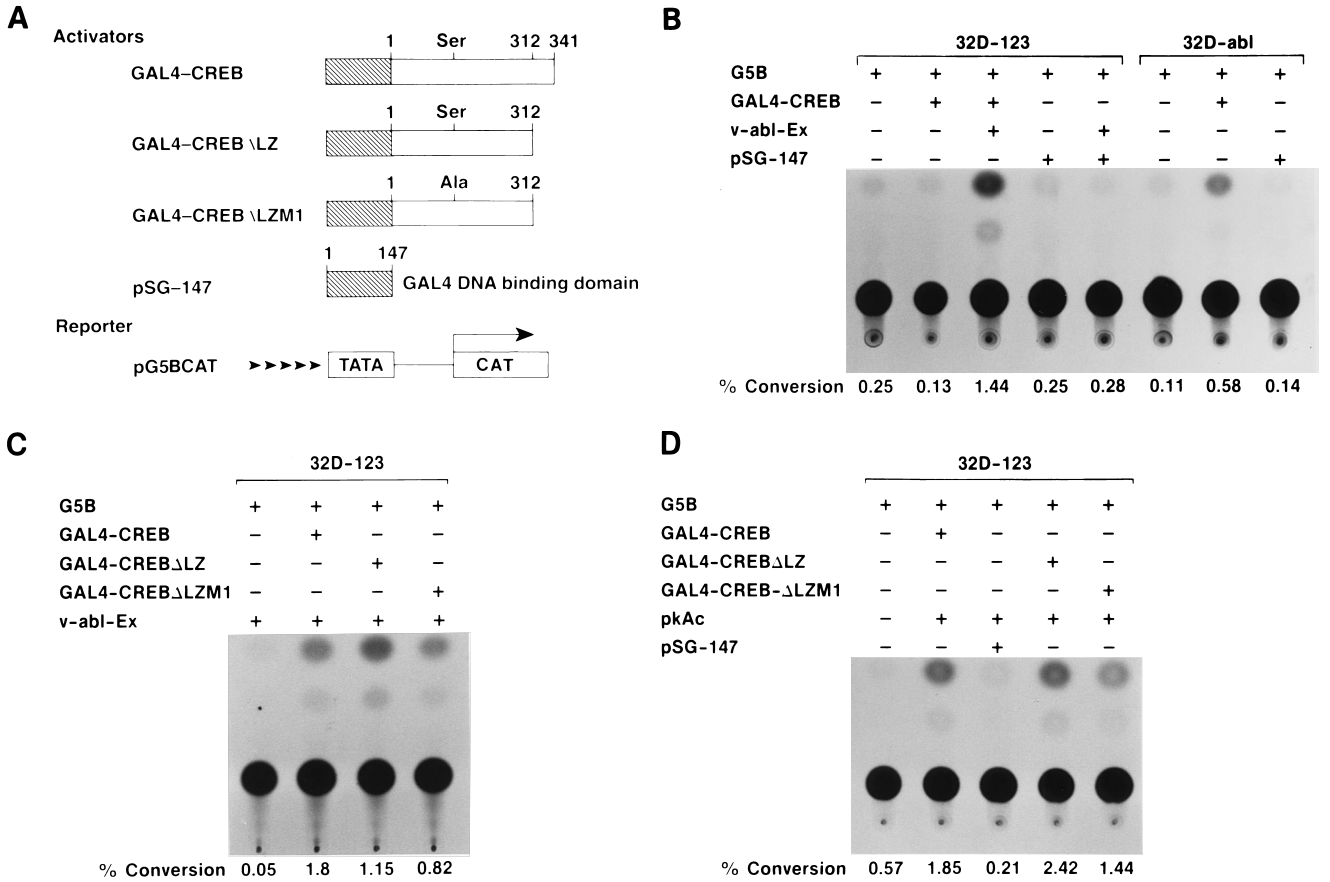

FIG. 3. v-Abl signals transcriptional activation via GAL4-CREB chimeric proteins in 32D myeloid cells. (A) Schematic diagram of the chimeric GAL4 activator proteins. The GAL4 DNA binding domain expression plasmid pSG-147 was fused to the entire CREB coding sequence (GAL4-CREB) or to CREB variants lacking the leucine zipper domain (GAL4-CREBΔLZ) or lacking the leucine zipper domain and containing a mutation of Ser-133 to Ala (GAL4-CREB-ΔLZM1). (B) Representative (CAT) assay of 32D-123 and 32D-abl cells cotransfected with 10 μg of G5B reporter plasmid, 10 μg of GAL4-CREB or pSG-147, and 15 μg of the v-*abl* expression vector (v-abl-Ex) or pUC18. (C) Transcriptional activities of the GAL4-CREB deletion mutants in 32D-123 cells. The myeloid cells were transfected with 10 μg each of the G5B reporter plasmid and the indicated GAL4 constructions in the presence of 15 μg of the v-*abl* expression vector. (D) Representative CAT assay of 32D-123 cells cotransfected with 10 μg each of the G5B reporter plasmid and the indicated GAL4 constructions or pSG-147 in the presence of 15 μg of the protein kinase A (catalytic subunit) expression vector (pkAc) or pUC18. CAT activity was measured 24 h later, and values were normalized as described in Materials and Methods. Results are representative of three independent experiments.

Earlier reports indicated that cAMP activation of GAL4-CREB proteins containing mutations of Ser-133 was reduced (45). To establish whether phosphorylation of this residue is functionally important for v-Abl-dependent activation, we used a mutant form of the GAL4-CREBΔLZ fusion protein in which Ser-133 was changed to an alanine (GAL4-CREBΔ LZM1). Activation of the GAL4-CREBΔLZM1 protein by transiently expressed v-Abl in 32D-123 cells was unaffected by the Ser-133 mutation, demonstrating that phosphorylation of this residue is not involved in the pathway of activation by v-Abl (Fig. 3C). Control experiments demonstrated that the catalytic subunit of protein kinase A activated the GAL4-CREB and GAL4-CREBΔLZ fusion proteins and, to a lesser degree, the GAL4-CREBΔLZM1 fusion protein but failed to

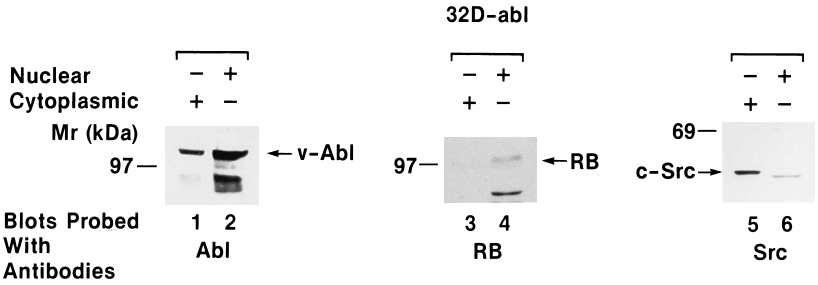

FIG. 4. Abl proteins are associated with the nuclear and cytoplasmic fractions of myeloid cells. Western blots of nuclear and cytoplasmic lysates of 32D-abl cells were probed with antibodies capable of recognizing the Abl (nuclear and cytoplasmic), retinoblastoma (RB) (nuclear), and c-Src (cytoplasmic) proteins. Results are representative of three independent experiments.

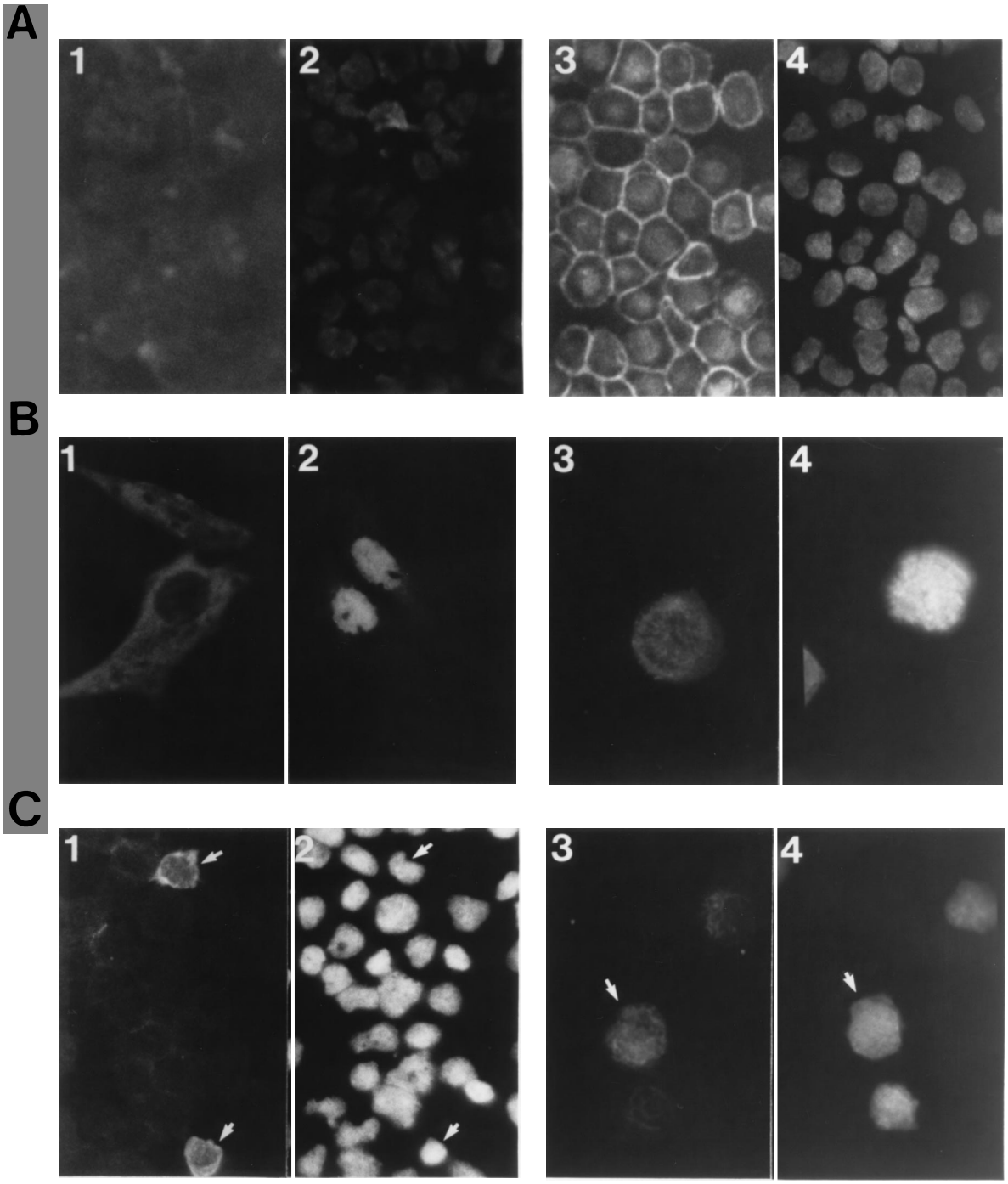

FIG. 5. Mapping of the p120 v-Abl NLS by indirect immunofluorescence. (A) Immunofluorescence photomicrographs of 32D-123 (panels 1 and 2) and 32D-abl (panels 3 and 4) cells stained with antibodies to Abl (panels 1 and 3) and DAPI (panels 2 and 4). (B) Shown are immunofluorescence photomicrographs of NIH 3T3 cells transfected with 1.5 μg of an IE175 gene (pGH115) with an internal deletion of the wild-type NLS (panel 1) and the IE175 gene with an internal deletion and containing the PKLLRR motif from p120 v-Abl (panel 2). The proteins were detected with a monoclonal antibody that specifically recognizes the C-terminal region of the protein and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G antiserum. Also shown are immunofluorescence photomicrographs of 32D-abl cells stained with antibodies to CREB (panel 3) and DAPI (panel 4). (C) Immunofluorescence photomicrographs of 32D-123 cells transfected with 15μg of Abl-M6 (panels 1 and 2) or Abl-FL (panels 3 and 4) and stained with antibodies to Abl (panels 1 and 3) and DAPI (panels 2 and 4). The corresponding nucleus (DAPI staining) and v-Abl immunofluorescence staining of individual 32D-123 cells is indicated by the arrows. Magnifications, ×400 (A) and ×1,000 (B and C).

activate the GAL4 (1 to 147) construction (Fig. 3D). Previous studies have similarly reported this partial activation of the GAL4-CREBΔLZM1 by protein kinase A, despite the mutation of the Ser-133 residue (45).

p120 v-Abl proteins are localized in the nuclear and cytoplasmic fractions of myeloid cells. We next investigated the distribution of the p120 v-Abl gene product in the 32D myeloid cells. Nuclear and cytoplasmic fractions of cellular extracts

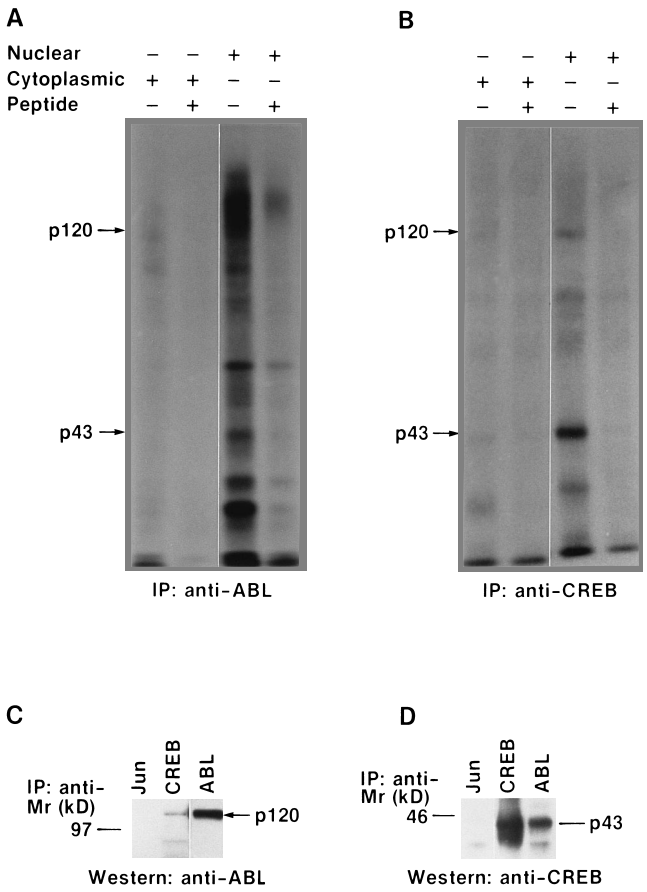

FIG. 6. v-Abl physically associates with CREB. Phosphorylated Abl proteins from 32D-abl cells can associate with phosphorylated CREBs. ^{32}P-labelled nuclear and cytoplasmic 32D-abl cell extracts were immunoprecipitated (IP) with anti-Abl (A) or anti-CREB (B) antibodies in the presence or absence of the appropriate competing peptide and were analyzed by SDS–14% PAGE followed by autoradiography. (C and D) Immunoprecipitation (with anti-c-*jun*, anti-CREB, or anti-Abl) and analysis of the v-Abl–CREB complex by SDS–7.5% PAGE and anti-Abl Western blotting (C) or SDS–14% PAGE and anti-CREB Western blotting (D). Results are representative of several independent experiments.

from 32D-abl cells probed by Western blot analysis with an anti-Abl antibody indicated the presence of the p120 v-Abl protein, and other smaller products of alternate splicing (p100 and p90 [42]) or degradation, at high levels in the nucleus and lower levels in the cytoplasm of 32D-abl cells (Fig. 4). The purity of the nuclear and cytoplasmic fractions was demonstrated by the appropriate reactivity of control antibodies against the nuclear retinoblastoma gene product and the cytoplasmic c-Src proteins (Fig. 4). The fact that the majority of the p120 v-Abl protein was found in the nuclear fractions of 32D-abl cells (Fig. 4) was unexpected because previous studies have indicated that the related viral p160 Abl protein is cytoplasmic (52).

By indirect immunofluorescence, the Abl proteins in 32D-abl cells were found to be associated with the nucleus and with the cytoplasmic membrane (Fig. 5A). While an NLS has been identified in the c-Abl protein (52), the corresponding region is absent in the p120 v-Abl protein. This led us to examine the p120 protein for a signal sequence that could target it to the nucleus. Inspection of the p120 coding sequence revealed several candidate peptide motifs that may function as an NLS, i.e., a short segment of protein containing a high basic amino acid content and one or more prolines. We found and tested two of these putative signals, PKRNKP (codons 339 to 344) and PKLLRR (codons 671 to 676), for the ability to establish a karyophilic phenotype in a heterologous, cytoplasmic protein. The ability of the second peptide, PKLLRR, to function as an independent NLS is shown in Fig. 5B. An expression vector encoding a mutant form of the herpes simplex virus type 1 IE175 protein which lacks the endogenous NLS (40, 47) was transfected into NIH 3T3 cells, where the mutant protein was observed to localize exclusively to the cytoplasm (Fig. 5B, panel 1). Insertion of the v-Abl PKLLRR motif at the site of the deletion restored the nuclear phenotype of the protein, demonstrating the ability of the PKLLRR motif to act as a nuclear targeting signal in a heterologous protein (Fig. 5B, panel 2). The NLS identified above was then mutated within the context of the wild-type v-Abl protein. Indirect immunofluorescence of 32D-123 cells transfected with an expression vector encoding a form of the v-Abl protein with an internal deletion of the PKLLRR NLS motif showed the mutant protein to be localized exclusively to the cytoplasm (Fig. 5C, panel 1). Control cells transfected with a vector expressing wild-type v-Abl demonstrated a predominantly nuclear phenotype of the protein (Fig. 5C, panel 3). In addition, immunofluorescence studies using anti-CREB antibodies demonstrated CREB to localized mostly to the nuclei of 32D-abl cells (Fig. 5B, panel 3).

Physical association between v-Abl and CREB. The demonstration that CREB is a target for activation by v-Abl together with the nuclear localization of the v-Abl protein caused us to study whether there is a physical interaction between v-Abl and CREB. To examine the possible association between these two proteins, we metabolically labelled 32D-abl cells with [^{32}P]P_i and analyzed the anti-Abl and anti-CREB immunoprecipitates by SDS–14% PAGE and immunoblotting. A 43-kDa protein was observed in the nuclear anti-Abl and anti-CREB precipitates but was absent when the immunoprecipitations were carried out in the presence of the appropriate competing peptide (Fig. 6A and B). The 43-kDa protein was also observed at very low levels in the cytoplasmic anti-Abl and anti-CREB immunoprecipitates. In addition, a 120-kDa protein was observed in the nuclear anti-Abl and anti-CREB precipitates but was absent when the corresponding Abl and CREB peptides were used during the immunoprecipitation as shown in Fig. 6A and B. The p120 band in the nuclear anti-Abl immunoprecipitates is not distinct because of the presence of multiple bands, and peptide inhibition appears to cause a general reduction in all of these bands. However, when all the data in Fig. 6 are considered, nuclear localization of the p120 v-Abl and CREB protein complex is strongly suggested. In addition, low levels of the p120 protein were observed in the anti-Abl immunoprecipitates from the cytoplasmic fractions. Identification of the 120-kDa protein as p120 Abl and the 43-kDa protein as CREB is shown in Fig. 6C and D. The p120 (v-Abl) protein was detected by Western blot analysis in an anti-CREB immunoprecipitate from whole cells, and CREB was similarly detected in an anti-Abl immunoprecipitate.

Protein mapping of the CREB-v-Abl interaction. To further support a physical interaction between the v-Abl and CREB, we next addressed the question of which regions of the proteins were involved in the interaction. Different segments of CREB were expressed in *E. coli* as fusion proteins with GST (Fig. 7B). The various GST-CREB fusion products were then analyzed for the ability to bind Abl proteins in 32D-123 and 32D-abl total cellular lysates (Fig. 7C and D). With lysates from 32D-abl cells, v-Abl bound GST fusion proteins that

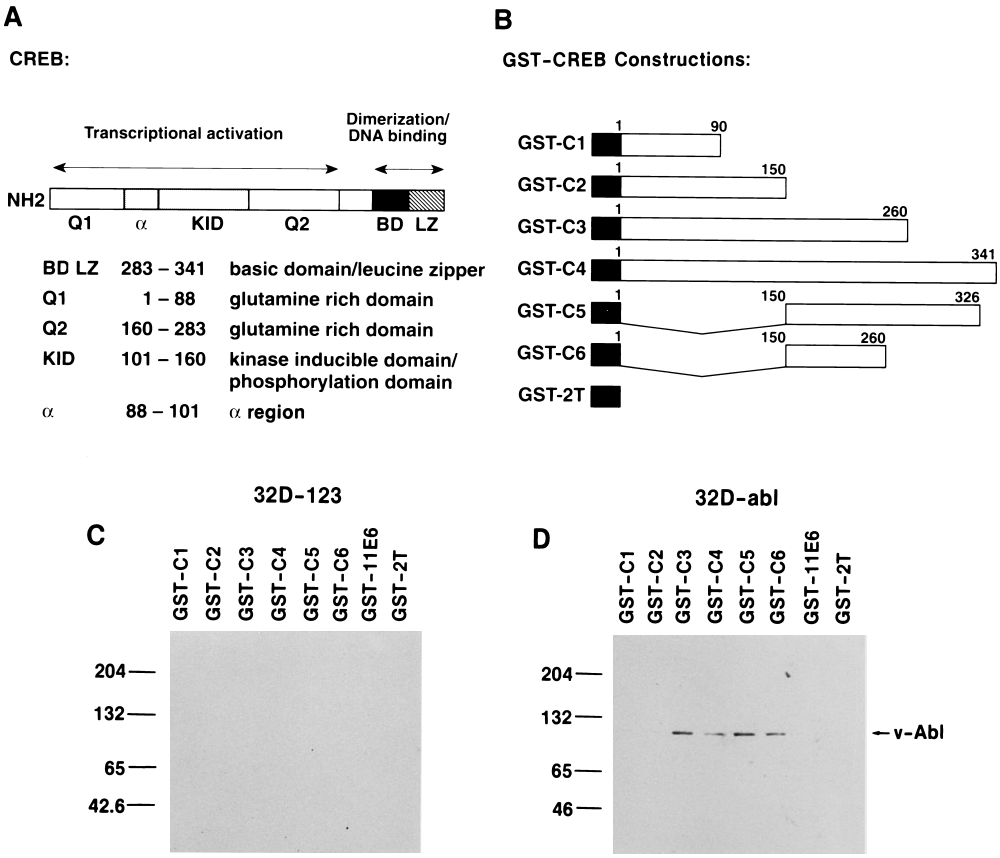

FIG. 7. v-Abl binds a domain of CREB containing the glutamine-rich Q2 motif. (A) Schematic representation of CREB indicating the amino acid numbers corresponding to each domain. (B) Diagrammatic representation of GST-CREB fusion proteins. GST-C1 contains only the glutamine-rich amino terminus (NH2) and the Q1 domain. GST-C2 contains only the Q1 and α domains and 10 amino acids of the KID domain. GST-C3 contains the Q1, α, and KID domains and part of the Q2 domain. GST-C4 contains the entire CREB protein. GST-C5 contains the Q2 glutamine-rich domain and the DNA binding domain. GST-C6 contains most of the Q2 domain. 32D-123 control (C) and 32D-abl (D) lysates were incubated with glutathione-agarose beads containing GST alone (GST-2T) or the GST-CREB fusion proteins indicated. Bound proteins were resolved by SDS–7.5% PAGE, analyzed by Western blotting with anti-Abl antibodies, and visualized by chemiluminescence. The position of the v-Abl protein is indicated.

contain either all of CREB (GST-C4) or only a region containing amino acids 150 to 260, which includes the Q2 glutamine-rich domain (GST-C5 and -C6). The Q2 glutamine-rich domain has been shown to be important in both the basal and cAMP-induced activities of CREB (5, 14). No interaction with v-Abl was observed with GST-2T alone or the fusion proteins with deletions in the C-terminal 251 (GST-C1) or 191 (GST-C2) amino acids of CREB. Thus, amino acids 150 to 260 of CREB are required and sufficient to bind p120 v-Abl. No detectable levels of c-Abl were bound to the different GST-CREB fusion proteins when lysates from 32D-123 cells were used (Fig. 7C). The Western blots shown in Fig. 7C and D were stripped and reprobed with a mixture of anti-CREB antibodies. Each of the fusion proteins exhibited the correct size and was present in approximately the same amount (data not shown). Together, these results demonstrate that the interaction between CREB and v-Abl occurs within a specific region of CREB that contains the Q2 glutamine-rich domain.

We next cotransfected GAL4-CREB fusion protein expression plasmids containing defined regions of CREB into 32D-123 cells in the presence or absence of the v-Abl protein (Fig. 8). Only the CREB fusion proteins that were demonstrated to physically interact with v-Abl (GST-C3, GST-C4, and GST-C6) activated the reporter plasmid, thus establishing a correlation between the ability of the v-Abl protein to associate with CREB and the activation of CREB-dependent transcription by the v-Abl protein.

To define the domain(s) of the v-Abl protein that interacts with the 110-amino-acid CREB peptide described above, we in vitro synthesized ^{35}S-labelled v-Abl peptides and analyzed them for the ability to bind to a GST-CREB fusion protein containing full-length CREB (GST-C4 [Fig. 7A and B]). Our studies indicate that while the amino-terminal (141-amino-acid) and the carboxy-terminal (371-amino-acid) regions of v-Abl were incapable of binding to the GST-CREB protein, a region containing both SH2 and the ATP binding domains of v-Abl (amino acids 241 to 513) bound the GST-CREB protein (Fig. 9B). We also examined the possibility that the SH2 domain or the ATP binding region could bind independently to CREB. These studies indicated only very weak binding between either the SH2 domain or the ATP binding regions of v-Abl and the GST-CREB protein (data not shown). Consequently, even though these studies indicate that SH2 and the ATP binding region of v-Abl (amino acids 241 to 427) are obligatory and sufficient to bind CREB, it is possible that a subdomain mapping between these two sites could be responsible for this association.

On the basis of the results of the above-described in vitro binding analysis, we constructed a series of v-*abl* expression vectors that contained mutations or deletions in the defined

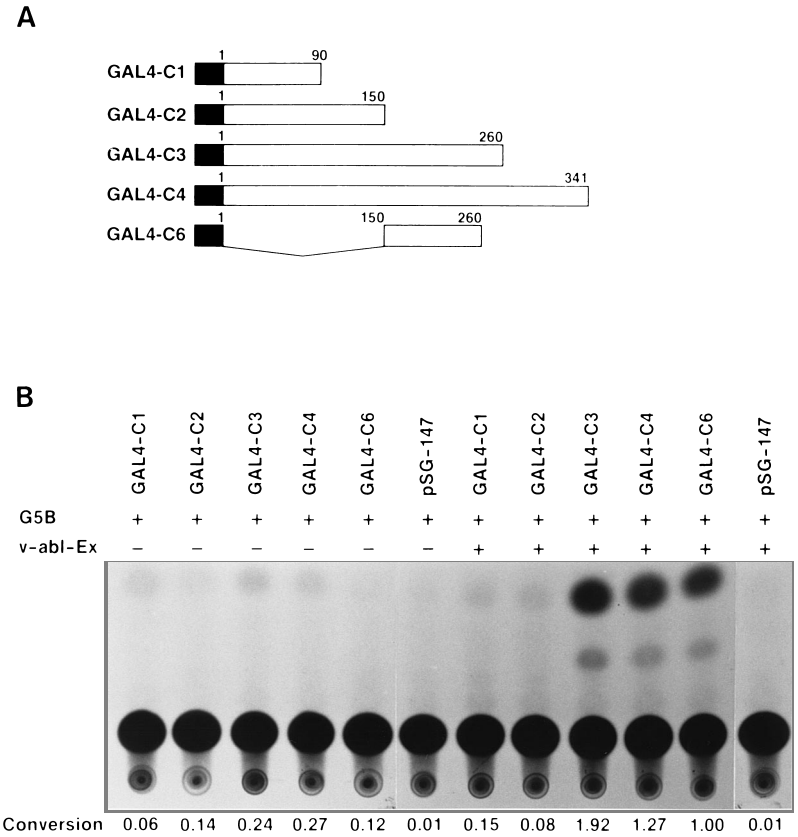

FIG. 8. Transactivation of GAL4-CREB fusion protein mutants by the v-*abl* expression vector (v-abl-Ex) is mediated through the domains defined in vitro. (A) Diagrammatic representation of the GAL4-CREB fusion proteins used in this experiment. The black areas represent the GAL4 DNA binding domain, and the white areas represent the coding region of CREB. (B) 32D-123 cells cotransfected with 10 μg each of the G5B reporter plasmid and the indicated GAL4 constructions or pSG-147 in the presence of 15 μg of the v-*abl* expression vector or pUC18. CAT activity was measured 24 h later, and values were normalized as described in Materials and Methods. Results are representative of three independent experiments.

CREB interaction domain. In addition, we included a 5-amino-acid deletion in the NLS, defined in Fig. 5 and diagrammed in Fig. 9C, to determine the requirement for a nuclear form of v-Abl in the activation of CREB. In agreement with the results of the v-Abl–CREB interaction domain mapping experiments that identified the SH2 and tyrosine kinase domains as being critical for the physical interaction with CREB, transactivation of GAL4-CREB by forms of v-Abl that contained either a point mutation (Abl-M1) or deletions (Abl-M7 and Abl-M9) in the ATP domain was nearly abolished (Fig. 9C). Moreover, restricting expression of v-Abl to the cytoplasm through deletion of the NLS also strongly inhibited the transactivation potential of the v-Abl protein, thus establishing the importance of a nuclear form of v-Abl for the activation of CREB (Fig. 9D). In vitro kinase assays performed with 32D-123 cells transfected with the different v-*abl* mutant expression vectors demonstrated that only the intact v-Abl protein and v-Abl with a deletion of the NLS (Abl-M1 and Abl-M6) possessed kinase activity (data not shown).

DISCUSSION

We have investigated the ability of the oncoprotein v-Abl to function as a transcriptional transactivator. Our studies have identified the transcription factor CREB as a mediator of the transcriptional effects of p120 v-Abl. A physical association between v-Abl and CREB proteins occurs; this association is mediated through protein domains such as the Q2 glutamine-rich domain of CREB and the SH2 or ATP binding domain of v-Abl.

The high level of transcriptional activity of CRE-containing genes in 32D-abl cells and the ability of transiently transfected v-Abl to increase the transcriptional activity of promoters containing intact CRE motifs in 32D-123 cells are evidence for the involvement of v-Abl in the activation of gene expression by CREB. CREB is a nuclear protein. Only extremely low steady-state levels of CREB are found in the cytoplasm (31, 57). Activation of CREB by v-Abl could occur through modification of CREB's DNA binding or transcriptional activation potential. It is important, then, that significant amounts of the v-Abl protein were detected in the nuclei of 32D-abl transformed cells. We have identified an independently functioning nuclear localization sequence (PKLLRR) within the v-Abl sequence. This sequence is completely conserved in both the murine c-Abl and p160 v-Abl proteins and thus represents a second nuclear translocation signal in these proteins. Another sequence, KKKKKMA, has been previously identified as a nuclear targeting signal in the murine c-Abl protein (52), and while this motif is also present in the murine p160 v-Abl protein, it has been deleted during the generation of the p120 form of the protein from p160 v-Abl (see Fig. 8A). Despite the presence of the pentalysine motif in p160 v-Abl, the protein remains cytoplasmic (52). It has been suggested that the nuclear translocation of the p160 protein is regulated by transforming mutations and that the pentalysine motif may confer only the potential for nuclear translocation (52). The possibil-

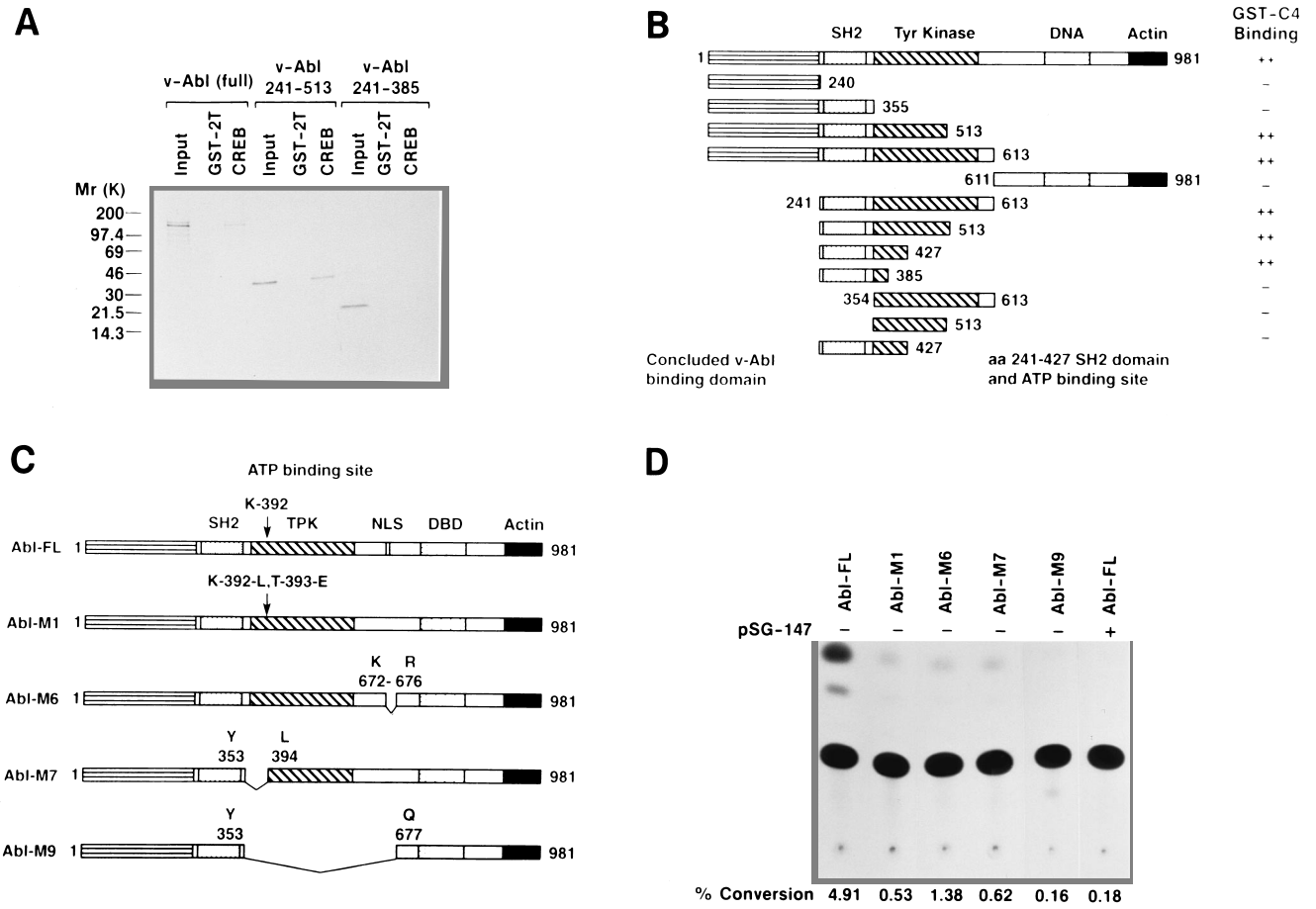

FIG. 9. CREB binds to a domain of v-Abl containing the SH2 domain and part of the ATP binding region. (A) In vitro-translated ^{35}S-v-Abl proteins were examined for the ability to bind CREB by incubation with GST-C4 (GST fused to full-length CREB) or GST-2T (GST alone) proteins immobilized on glutathione-agarose beads. The regions of v-Abl that were studied include full-length v-Abl (amino acids 1 to 981) (full), amino acids 241 to 513, and amino acids 241 to 385. (B) Diagrammatic comparison of the p120 gag-Abl protein. Characterized regions are indicated as follows: SH2, tyrosine kinase domain including the ATP binding region (Tyr kinase), DNA binding domain (DNA), and actin binding domain (Actin). (C) Diagram of the v-Abl deletion constructs. Sequences are indicated beginning with the first amino acid of the Gag portion of v-Abl. The tyrosine kinase domain (TPK), nuclear localization signal (NLS), DNA binding domain (DBD), and actin binding domain (Actin) are also shown. (D) 32D-123 cells cotransfected with 10 μg each of the G5B reporter plasmid and the full-length GAL4-CREB construction or pSG-147 in the presence of 15 μg of the indicated v-Abl expression mutants or pUC18. CAT activity was measured 24 h later, and values were normalized as described in Materials and Methods. Results are representative of three independent experiments.

ity of a second NLS in the p160 protein has been postulated before, on the basis of the partial nuclear character of a mutant p160 protein that lacks the pentalysine motif (52). The presence of a nuclear targeting sequence within p120 v-Abl, despite the loss of one such signal during the generation of the p120 form from p160 v-Abl, suggests a possible selection pressure for nuclear translocation in the biology of both of these viral proteins.

We have demonstrated that the p120 v-Abl protein can form stable, immunoprecipitable complexes with cellular CREB and that the functional activation of CREB by p120 v-Abl is dependent on this interaction. By using defined regions of human CREB fused to the DNA binding domain of the yeast activator GAL4, a 110-amino-acid region of CREB that includes the glutamine-rich Q2 domain was shown to be sufficient for specific association with the v-Abl protein. The Q2 domain has been shown to be important for the transactivation potential of CREB and has also been reported to be one of the two regions (amino acids 41 to 86 and 165 to 252) that mediate basal and cAMP-stimulated transcription (36). In addition, Q2 has been shown to function together with TFIID to act as a potential constitutive activator in vitro (11). Protein-protein interactions between CREB and other transactivators have been shown to occur through the leucine zipper motif (22), but our studies indicate that fusion proteins lacking this motif retain the ability to form complexes with v-Abl. Moreover, v-Abl does not appear to contain the coiled-coil motif necessary for an interaction through the leucine zipper domain of CREB.

Using defined regions of the v-Abl protein, we have demonstrated the interaction of the CREB protein with a region containing the SH2 domain and the ATP binding region of the v-Abl protein. While protein-protein interactions involving SH2 domains of a variety of proteins involved in signal transduction are known (reference 27 and references therein), the postulated direct interaction with transcription factors is novel. Moreover, the ATP binding region of the cellular protein c-Abl has been shown to associate with the retinoblastoma gene product (56). Since v-Abl shares these regulatory domains with c-Abl, it is possible that c-Abl could also interact with CREB and alter its transcriptional activity. However, because the concentration of c-Abl in the 32D-123 cells is extremely low, it has been difficult to directly study its potential transactivation ac-

tivity. Nevertheless, the ability of transiently transfected c-Abl to increase the transcriptional activity of promoters containing intact CRE motifs in 32D-123 cells provides evidence for the involvement of c-Abl in the activation of gene expression by CREB. The present studies with the GAL4-CREB fusion proteins demonstrated that the mechanism by which v-Abl activates CREB is unlikely to be the same as the cAMP-dependent pathway, since mutation of Ser-133 had no effect on CREB transactivation activity. The presence of other potential phosphorylation sites in CREB (5) could provide alternate means by which v-Abl could regulate CREB activity. However, immunoprecipitation studies involving in vivo ^{32}P-labelling of 32D-123 and 32D-abl cells showed no difference in the levels of phosphorylated CREB (data not shown). Nevertheless, it is possible that the conditions used in our studies could not detect a transient difference in the phosphorylation of CREB or a change in the phosphorylation state of a minor fraction of total cellular CREB. For example, only the promoter-bound fraction of CREB may be subjected to kinase activity by v-Abl to increase its transactivation potential. Furthermore, if such kinase activity is cell cycle regulated, as has been previously shown for c-Abl kinase activity during the S phase of the cell cycle (56), the experimental conditions used may not detect the difference in the phosphorylation state of CREB.

Another conceivable mechanism by which v-Abl can activate CREB could involve the targeting of the v-Abl protein directly to promoters containing CRE motifs via its association with CREB. A comparable model has been proposed for the association between the herpes simplex virus transactivator VP16 and octamer transcription factor 1 (OTF-1) (50). Like Abl, VP16 cannot bind to DNA independently but rather is targeted to appropriate promoters by a protein-protein interaction with the ubiquitous octamer transcription factor 1. Interestingly, recent studies have demonstrated the ability of c-Abl to phosphorylate the carboxy-terminal domain of the catalytic subunit of RNA polymerase II (3, 56). The phosphorylation of the carboxy-terminal domain has been proposed to facilitate the transition from initiation to transcriptional elongation (9). It is conceivable that CREB targets v-Abl to the transcription initiation complex, thus facilitating v-Abl's phosphorylation of the carboxy-terminal domain.

Our present study indicates that p120 v-Abl physically associates with CREB, but we cannot exclude the possibility that the interaction between CREB and v-Abl is mediated through one or more accessory proteins. In this regard, it has recently been shown that the adenovirus E1a protein binds to a protein, p300, that is 95% homologous to the CREB-binding protein postulated to function in the normal activation of CREB by cAMP (2). Thus, it is possible that this or a similarly acting protein may be involved in the activation of CREB by p120 v-Abl.

In almost all cases of chronic myelogenous leukemia (39, 43) and in 10 to 20% of cases of acute lymphoblastic leukemia (33, 39) a Philadelphia chromosome is present; this chromosome involves activated versions of the c-*abl* proto-oncogene. In humans and mice the activated *abl* genes are most often found in hematopoietic stem cell tumors, and in mice and cats it has been recovered in transforming retroviruses (39, 44). Recombination events similar to the event that generated Abelson murine leukemia virus in mice have been postulated to occur frequently in human chronic myelogenous leukemias (39). Understanding the mechanism by which activated Abl signals transformation and leukemogenesis is critical for the control of these neoplasias. Initial studies identified the growth-stimulatory activity of v-Abl (38) to be associated with the induction of serum-responsive genes such as c-*fos*, c-*jun*, and c-*myc* and to be dependent on the cell context (38). Our results demonstrate that v-Abl stimulatory activity can also be associated with the regulation of CRE-containing genes. The demonstration that v-Abl physically interacts with CREB and transactivates CRE-containing promoters opens a new window for the study of potential interactions with other transcription factors and may advance the understanding of the function of Abl and CREB in the context of cellular physiology, transformation, and neoplasia.

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