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The *ras*-Related Gene *rhoB* Is an Immediate-Early Gene Inducible by v-Fps, Epidermal Growth Factor, and Platelet-Derived Growth Factor in Rat Fibroblasts

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A set of genes is rapidly inducible when quiescent fibroblasts are stimulated by growth factors or by the activation of temperature-sensitive retroviral protein-tyrosine kinases. Most of these so-called immediate-early genes were cloned by differential cDNA hybridization. DNA sequence analysis identified many of them as putative members of the growth factor or of the transcription factor gene family, suggesting a role in signal transmission during the G_0-to-G_1 transition. In this study, we identified one of the genes that are rapidly inducible by the retroviral protein-tyrosine kinases v-Src and v-Fps of Rous sarcoma virus and Fujinami sarcoma virus, respectively, as the *rhoB* gene, a member of the *ras* gene superfamily whose products are GTP-binding proteins. *rhoB* is transiently activated at the transcriptional level by v-Fps and by epidermal growth factor. Its labile RNA is inducible in the presence of cycloheximide but not of actinomycin D. *rhoB* is strongly induced by epidermal growth factor and by platelet-derived growth factor both in subconfluent, serum-starved and in density-arrested Rat-2 fibroblasts. Fetal calf serum is a poor inducer, particularly in density-arrested cells, and phorbol esters do not increase *rhoB* expression at all. These data suggest that *rhoB* is inducible by protein-tyrosine kinases through a pathway not involving the activation of protein kinase C. Neither the closely related *rhoC* and *rhoA* genes nor the distantly related c-H-*ras* gene is rapidly inducible by mitogens. Thus, *rhoB* is the first known member of the small GTP-binding proteins among the immediate-early genes.

The biochemical mechanisms of growth factor- and oncogene-stimulated DNA synthesis are not well understood. One pathway of signal transduction involves transmembrane receptors with intrinsic protein-tyrosine kinase (PTK) activity. Binding of a ligand, such as epidermal growth factor (EGF) or platelet-derived growth factor (PDGF), leads to an activation of the respective receptor PTK that is necessary for the stimulation of the numerous metabolic events leading to DNA synthesis. Another mitogenic pathway involves the activation of the protein kinase C (PKC), either by direct interaction with tumor promoters like tetradecanoyl phorbol acetate (TPA) or by second messengers like diacylglycerol and 1,4,5-inositol triphosphate, which are generated through the stimulation of an inositide phosphate-specific phospholipase by certain regulatory peptides and neurohormones (for reviews, see references 54, 69, and 72).

A number of so-called immediate-early genes are rapidly and transiently activated at the transcriptional level when quiescent cells are stimulated by mitogens or by oncogenes to enter the cell cycle (1, 32, 36, 37, 65). The functions of most of these genes remain to be determined. Some of them, like c-*fos*, *fosB*, *fra-1*, c-*jun*, *jun-B*, NGFI-A (also known as *egr-1*/*Krox-24*/*zif268*), *Krox-20*/*egr-2*, and NGFI-B/N10/*nur77*, encode DNA-binding proteins (2, 10, 15, 18, 20, 21, 31, 33, 38, 43, 46, 55, 58, 74). Two short DNA sequences termed the serum response element (SRE) and TPA response element (TRE) have been identified in the promoter regions of many of these genes. The SRE and TRE serve to integrate the transcriptional response to serum growth factors and to activators of PKC (2, 9, 11, 14, 17, 24, 25, 27, 51, 58, 62, 67, 68).

Two immediate-early genes, named KC and JE, show a distinct pattern of response to mitogens; in comparison with the c-*fos* gene, their transcriptional induction by PDGF is more prolonged and no stimulation by TPA is observed (19, 48). The promoter region of the KC gene has not been analyzed; the JE gene promoter lacks an SRE sequence, and an element resembling a TRE is apparently not functional in rodent fibroblasts (66), suggesting that the signal transduction through receptor PTKs involves undefined DNA sequence elements.

The retroviral oncogenes v-*src* and v-*fps* of Rous sarcoma virus and Fujinami sarcoma virus, respectively, both encode PTKs that are localized predominantly at the inner surface of the plasma membrane (50, 60, 71). To study the function of this class of PTKs in the signal transduction process, we had cloned previously a group of genes that are induced rapidly in rat fibroblasts following activation of a temperature-sensitive (*ts*) mutant of the v-*src* PTK by shift to the permissive temperature (32).

In this study, we characterized one of these genes, previously termed 7VI-2 (32), and showed that it encodes the *rhoB* protein, a member of the *ras* superfamily of GTP-binding proteins. We show here that *rhoB* constitutes the first example of an immediate-early gene within this group of genes. The *rhoB* gene is inducible by EGF, PDGF, and the v-*fps* PTK but not upon activation of PKC. In Rat-2 cells, it is regulated differently from all other immediate-early genes that were analyzed in parallel, including c-*fos*, NGFI-A, c-*jun*, and KC. Thus, the v-*fps* PTK shares a novel pathway of signal transduction with receptor PTKs for the activation of a novel immediate-early gene.

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MATERIALS AND METHODS

Cell lines and cell culture. Rat-2 fibroblasts and the Rat-2 clone 2491, expressing a *ts* mutant of Fujinami sarcoma

virus, were obtained from Gerry Weinmaster (Salk Institute) (70). Normal rat kidney (NRK) cells and LA23 NRK cells were a gift of Peter Vogt (University of Southern California) to Jon Cooper (Salk Institute). All cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) in a humidified 10% CO_2–90% air atmosphere at 37°C. New cultures were started from frozen stocks every 6 to 8 weeks. To render cells quiescent by serum starvation, they were plated at 39.5°C (nonpermissive temperature) at 10^4 cells per cm^2 and grown for 5 to 6 days. They were then rinsed with DMEM and incubated for a further 48 h in DMEM supplemented with 0.2% FCS. Stimulation was achieved by shift to 34°C (permissive temperature). Rapid temperature shift was achieved by taking the culture dishes from a 39.5°C incubator and floating them for 2 min in a 34°C water bath before transferring them to a 34°C incubator. In some experiments, Rat-2 cells were grown to 70% confluence in 10% FCS, and after a rinse with DMEM, they were incubated for 20 h in DMEM without FCS. To achieve conditions of density arrest, 2491 and Rat-2 cells were plated at 2×10^4 to 3×10^4 cells per cm^2 and grown for 4 days. After a change of medium, they were incubated for another 48 h.

Mitogenic stimulation. EGF (receptor grade) was from Calbiochem (La Jolla, Calif.), PDGF (the c-*sis* B-chain dimer) was purchased from Amgen (Thousand Oaks, Calif.), bovine pancreatic insulin was from Sigma (St. Louis, Mo.), human basic fibroblast growth factor (bFGF) was a gift from Andrew Baird (Whittier Institute, La Jolla, Calif.), and human transforming growth factor β1 (TGF-β1) was a gift from Bill Boyle (Salk Institute). Vasopressin was kindly provided by Jean Rivier (Salk Institute), and dibutyryl-cyclic AMP (db-cAMP) was a gift of Gary Glenn (Salk Institute) and was biologically tested by differentiation of F9 cells. Actinomycin D was from Boehringer Mannheim (Indianapolis, Ind.); forskolin was from Calbiochem, TPA was from L.C. Services (Woburn, Mass.); phorbol dibutyrate (PdBu), dexamethasone, and cycloheximide were purchased from Sigma. The mitogenic peptides were dissolved as suggested by the suppliers and stored in aliquots to avoid repeated thawing. Vasopressin, db-cAMP, cycloheximide, and actinomycin D were dissolved in H_2O; dexamethasone, forskolin, and TPA were stored in ethanol; PdBu was stored in dimethyl sulfoxide. For treatment of quiescent cells, stock solutions were added to the cell culture media without change of media or of temperature. The following final concentrations were used: EGF, PDGF, and bFGF, 20 ng/ml; insulin, 1 μg/ml; TGF-β1, 2.5 ng/ml; TPA, 50 ng/ml; PdBu, 200 ng/ml; dexamethasone, 1 μM; vasopressin, 100 nM; forskolin, 20 μM; db-cAMP, 100 μM; cycloheximide, 20 μg/ml; and actinomycin D, 5 μg/ml. The added solutions constituted 1% or less of the volume of medium and did not result in changes of RNA levels. FCS was added to a final concentration of 10%.

Isolation of cDNA clones. A rat λZAP cerebellum cDNA library (1.5×10^6 independent clones), a gift of Jim Boulter (Salk Institute), was screened by hybridization as follows. Phage growth and preparation of replica plaque lifts onto nylon membranes (Amersham, Arlington Heights, Ill.) was done by standard techniques (41). The *Pst*I-*Eco*RI insert of cDNA 7VI-2 (32) was labeled by random priming, using a commercial kit (Boehringer Mannheim). Hybridization was performed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C; membranes were washed in 6× SSC–0.5% sodium dodecyl sulfate (SDS) at 65°C and finally in 0.1× SSC–0.1% SDS at 65°C for 30 min. The longest cDNA, clone 7-18, was used to generate DNA fragments that served as probes to rescreen the same library resulting in the isolation of clone 7-18-8K. The 0.54-kb *Sac*I-*Bgl*I fragment from the coding region of 7-18-8K was used to isolate related sequences by hybridization of the same library lift at 60°C in 6× SSC. Final washes were performed in 1× SSC–0.5% SDS at 50°C. Forty-seven clones were isolated, and *rhoA* and *rhoC* cDNAs were identified by restriction enzyme mapping and partial sequence analysis.

DNA sequencing. Libraries of random, sonicated DNA fragments were inserted into m13 mp19 according to Bankier et al. (4). Clones were sequenced by the chain termination method of Sanger et al. (59), using a commercial T7 polymerase kit (U.S. Biochemical Corp., Cleveland, Ohio). The sequence was read from independent clones at least three times in each direction. Difficult regions were sequenced in both directions with an inosine mix substituting for the guanosine mix. Sequence data were compiled and analyzed by using programs from Intelligenetics, Staden, and the University of Wisconsin Genetics Computer Group.

RNA extraction, Northern (RNA) blots and RNA dot blots. Total RNA was extracted from cell cultures according to the procedure of Chomczynski and Sacchi (16). Individual 15-cm tissue culture plates were rapidly rinsed with ice-cold isotonic Tris buffer (pH 7.4) and quickly drained, and the cells were lysed with 3 ml of the guanidine isothiocyanate solution at room temperature. Then 10 to 20 μg of RNA was denatured by treatment with formaldehyde and formamide and run on 1% agarose-formaldehyde gels as described by Maniatis et al. (41). The nucleic acids were transferred overnight by capillary action to Nytran membranes (Schleicher & Schuell, Keene, N.H.) in 10× SSC. The RNA was cross-linked to the membranes by UV irradiation as suggested by the manufacturer. The transfer of RNA was controlled by staining on the membranes with methylene blue. Probes were labeled with [γ-^{32}P]dCTP (3,000 Ci/mmol; Amersham) by primed-cut labeling, using m13 mp19 clones carrying the cDNA inserts of the 9I-6 (*fos*), 9I-1 (KC), 9II-6 (NGFI-A), or 7VI-2 (*rhoB*) plasmids (32). Random DNA fragments from the 3′ noncoding regions of the *rhoC* and *rhoA* genes were identified by DNA sequencing (see above) and labeled similarly. A *Hin*dIII-*Pst*I fragment from the Rasheed sarcoma provirus (a gift of Candy Haggblom, Salk Institute) was labeled by random-primed synthesis and used as a probe for the rat c-H-*ras* gene. Filters were hybridized for 36 h at 42°C in 6× SSPE (41)–0.5% SDS–5× Denhardt's solution–100 μg of sonicated and denatured salmon sperm DNA per ml–50% deionized formamide. Filters were washed with 0.2× SSC–0.2% SDS at 60°C and exposed at −70°C to presensitized Kodak XAR film with an intensifying screen. Probes were stripped from filters by incubation at 95°C in 10 mM Tris-HCl (pH 8)–1 mM EDTA–0.1% SDS three times for 5 min each time.

For RNA dot blots, 3 μg of total RNA was denatured with formamide and formaldehyde and bound to Nytran membranes in 10× SSC by filtration through a 96-well manifold apparatus (Schleicher & Schuell). Filters were processed as described above.

Nuclear run-on transcription analysis. Nuclei were prepared from four 150-mm dishes of cells per time point, and nuclear run-on transcription assays were performed according to Almendral et al. (1), using 40 μl of [γ-^{32}P]UTP (800 Ci/mmol; New England Nuclear, Boston, Mass.) per assay. Labeled RNA from each time point was hybridized to 5 μg of the following DNAs: Bluescript SK, used as vector control; *rhoC*, *rhoA*, and *rhoB* cDNAs from rat cerebellum of 1,200,

700, and 2,200 bp, respectively, in Bluescript SK; c-*jun* (a 600-bp fragment from the 5′ end of the murine cDNA in pIBI76 [35]) (a gift of Bill Lamph, Salk Institute); β_2-microglobulin (a 600-bp *Pst*I fragment of a murine cDNA in pBR322); c-*fos* and NGFI-A (plasmids 9I-6 and 9II-6, respectively, containing about 260 bp of rat cDNAs each in pIBI76 [32]); H-*ras* (the *Hin*dIII-*Pst*I fragment of the Rasheed sarcoma virus in pGEM3; a gift of Candy Haggblom); and 4VI-1 and 5IV-7 (rat cDNA plasmids containing inserts of 140 and 300 bp, respectively, in pIBI76 [32]). All plasmids had been denatured by incubation for 15 min in 0.5 M NaOH at 37°C, neutralized with an equal volume of 2 M ammonium acetate (pH 4.5), and bound in 10× SSC to Nytran membrane filters by filtration through a 96-well manifold apparatus (Schleicher & Schuell).

RESULTS

Identification of 7VI-2 as the *rhoB* gene, a member of the *ras* family of GTP-binding proteins. The 7VI-2 cDNA had been cloned in a screen for genes inducible by the v-*src* protein, the oncogenic PTK encoded by Rous sarcoma virus (32). It contained 300 bases from an RNA of 2.4 kb and showed no significant homology to DNA sequences present in various data bases. To assign a function to the 7VI-2 gene, we isolated a more complete cDNA, named 7-18-8K, by screening a rat cerebellum cDNA library with the 7VI-2 probe. Four restriction fragments, together representing the full-length 7-18-8K clone, each identified the same RNA of 2.4 kb in samples from rat brain and rat fibroblasts (data not shown; see below).

The sequence of the 7-18-8K cDNA reveals an open reading frame for a protein of 196 amino acids (Fig. 1), which is preceded by two in-frame stop codons and a single consensus initiation codon. The two other frames within the predicted noncoding region contain no stop or initiation codons, but four stop codons each are present within the predicted coding region. The 7VI-2 cDNA is part of the predicted 3′ untranslated sequence of 7-18-8K, confirming the hybridization data. The predicted protein sequence of the 7VI-2 gene is identical to the one encoded by the human *rhoB* gene (also called *rho6* [13]); the corresponding DNA sequences are 93% identical. Thus, the 7VI-2 gene is the rat homolog of the human *rhoB* gene. *rhoB*, and the closely related genes *rhoC* (also called *rho9*) and *rhoA* (also called *rho12*) (13, 73), are members of the *ras* superfamily of GTP-binding proteins, which were originally cloned from a human T-cell cDNA library by virtue of their homology to the *ras* gene product (40). Their role in the physiology of mammalian cells has not been determined yet, and no analysis of their transcriptional regulation has been performed.

The 5′ noncoding sequence of the rat *rhoB* gene as determined from clone 7-18-8K consists of at least 240 bases with an unusually high G+C content of 80%. The *rhoB* cDNA sequence of 2,180 bases accounts for most of the 2.4 kb of the *rhoB* mRNA. The precise 5′ and 3′ ends of the gene as well as the position of the poly(A) addition signal remain to be identified. Except for 31 bases of 3′-flanking sequences, no data are available for the noncoding regions of the human *rhoB* gene, explaining the failure to identify the gene by data base searches with the sequence of the 7VI-2 cDNA.

To compare the *rhoB* gene with the related rat *rhoA* and *rhoC* genes, we isolated their cDNAs from the same cDNA library (see Materials and Methods). DNA fragments from the 5′ and 3′ noncoding regions of the *rhoB* cDNA and from the 3′ noncoding regions of the *rhoC* and *rhoA* clones did not cross-hybridize and detected rat fibroblast RNAs of 2.4, 1.5, and 2.2 + 1.8 kb, respectively (see below).

Selective induction of *rhoB* but not of *rhoC* and *rhoA* by PTKs. To characterize the regulation of the *rhoB* gene by a retroviral nonreceptor PTK, we chose 2491 cells, a clone of Rat-2 fibroblasts expressing a *ts* mutant of the v-*fps* oncogenic PTK (70). A change in v-*fps* PTK activity occurs within the first hour after shift from the nonpermissive temperature of 39°C to the permissive temperature of 34°C. Northern blot analysis of cellular RNA at various time points after shift of quiescent, serum-deprived 2491 cells from 39 to 34°C shows that this treatment induced *rhoB* RNA within 15 min, with maximal expression after 60 min and a decline thereafter (Fig. 2A). Only a minimal elevation of *rhoB* expression was observed in identically treated Rat-2 cells (Fig. 2B), establishing that activation of the v-*fps* PTK and not temperature shift per se leads to the induction of *rhoB* in 2491 cells. Similar results were obtained with LA23 NRK fibroblasts, a cell line expressing a *ts* mutant of the v-*src* protein (data not shown).

The signal transduction pathways involved in the rapid induction of *rhoB* by defined mitogens were studied in 2491, Rat-2, and NRK fibroblasts that had been rendered quiescent by serum deprivation (Fig. 2 and data not shown). EGF and PDGF were the most efficient activators of *rhoB* in all three cell lines tested, being at least as efficient as the v-*fps* PTK or FCS (see also below). No response of *rhoB* was found with any other mitogen tested, including growth factors that activate receptor PTKs, such as insulin and bFGF, with any activator of PKC tested, such as the phorbol ester PdBu (Fig. 2C), TPA (see below), or vasopressin, or with agents acting through protein kinase A, such as forskolin and db-cAMP. No induction of *rhoB* expression occurred after treatment with dexamethasone or with double-stranded RNA. The activation by EGF was not influenced by additional treatments with either PdBu or FCS (Fig. 2C), insulin, or TGF-β1. Similarly, there was no influence of simultaneous treatments with these mitogens on the induction by the v-*fps* PTK in 2491 cells. In summary, in rat fibroblasts *rhoB* is selectively inducible by activated PTKs but apparently not by activation of protein kinase A or C.

In contrast to *rhoB*, *rhoC*, *rhoA*, and c-H-*ras* were not rapidly induced in any of the experiments described above (Fig. 2 and data not shown). This result is surprising given that the three *rho* genes are more than 90% identical in protein sequence (13, 73). The distantly related *ras* genes are not transcriptionally activated by short-term mitogenic stimulation of murine fibroblasts (28, 45), but an increase of c-H-*ras* RNA occurs after stimulation with FCS for several hours in Rat-2 cells (not shown) and in BALB/c 3T3 cells (39).

***rhoB* is an immediate-early gene.** The rapid and transient induction of the *rhoB* RNA by the v-*fps* PTK (Fig. 2) is reminiscent of the regulation of a group of so-called immediate-early genes by mitogens. Their transient expression results from a rapid and transient transcriptional activation of unstable RNAs (1, 36, 37). To determine whether *rhoB* is similarly regulated, we stimulated quiescent Rat-2 cells for various times with EGF and analyzed the cellular RNAs by the dot blot procedure (Fig. 3A). The induction by EGF was transient, showing a peak after 30 min, which fell back to near the baseline level 2 h after stimulation. Similar results were obtained with PDGF and FCS (not shown). Simultaneous treatment with EGF and cycloheximide led to a superinduction of *rhoB*. Analogous experiments with 2491

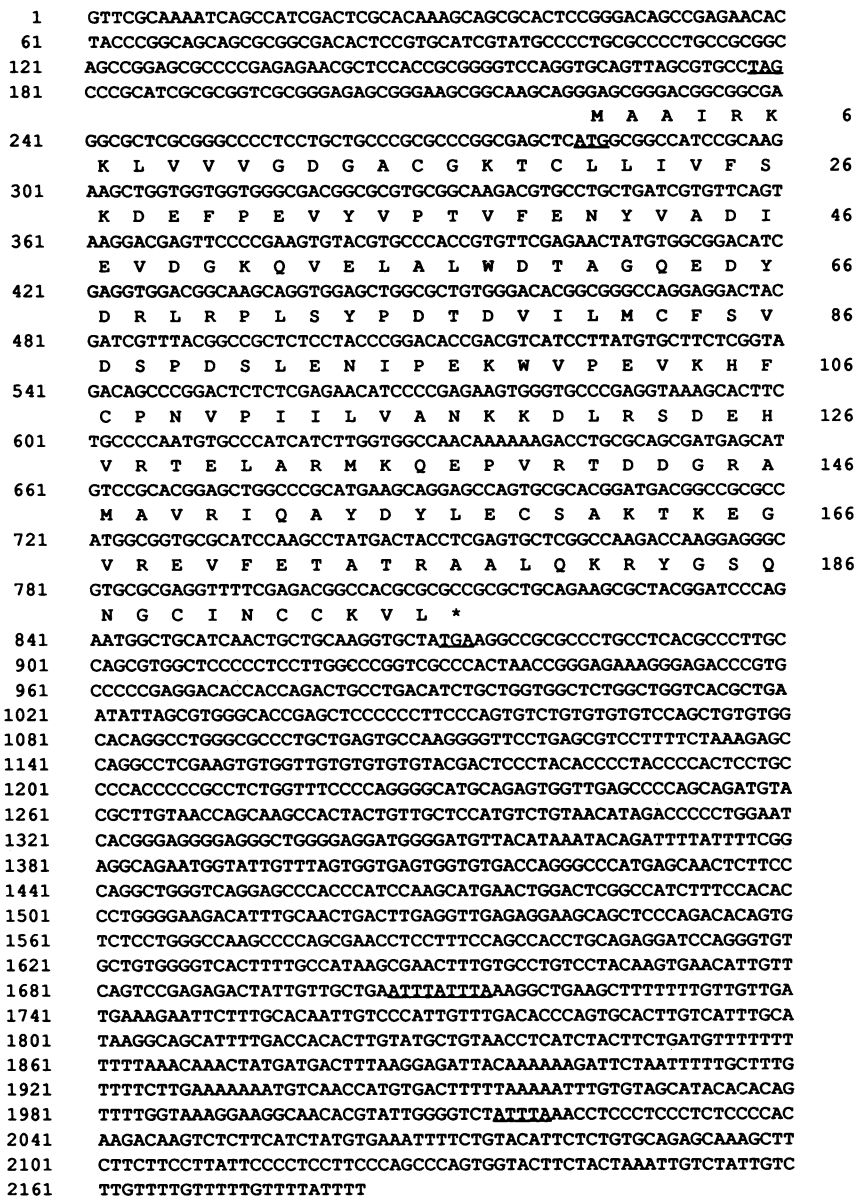

FIG. 1. Rat *rhoB* cDNA and protein sequences. The nucleotide and deduced amino acid sequences of the *rhoB* gene of the rat are shown. Nucleotide positions are given on the left, and the deduced amino acid positions are given on the right. The putative instability sequences (61) are underlined. An in-frame stop codon upstream from the initiation codon, the initiation codon, and the stop codon are underlined.

cells were inconclusive, since incubation with cycloheximide itself resulted in a substantial increase of *rhoB* expression (data not shown). In contrast to its induction in the presence of inhibitors of protein synthesis, the EGF-mediated induction was prevented by a pretreatment for 15 min with actinomycin D, an inhibitor of mRNA synthesis (Fig. 3B). The transcriptional control of *rhoB* RNA induction was analyzed further by run-on experiments with isolated nuclei. Its transcription was transiently increased as soon as 15 min after stimulation of quiescent Rat-2 cells with EGF and 30 min after activation of the v-*fps* PTK in 2491 cells (Fig. 4A and B). The rate of transcription returned to baseline levels within 1 to 2 h after induction. The 5- to 10-fold increase of the transcriptional rate in isolated nuclei corresponded to the increase of RNA levels in intact cells, and its peak preceded the maximum of RNA expression by about 15 min. Thus, no other level of control may be involved in the induction of *rhoB*. To explain the transient nature of this process, we analyzed the stability of *rhoB* RNA. By Northern blot analysis, we determined the amounts of the *rho* RNAs at different time points after blocking their synthesis by actinomycin D (Fig. 4C). As predicted, the half-life of the *rhoB* RNA is short, between 15 and 30 min. In contrast, *rhoC* and *rhoA* encode relatively stable RNAs and resemble housekeeping genes with a low rate of transcription in both quiescent and mitogenically stimulated cells.

***rhoB* is regulated differently from the c-*fos*, NGFI-A, c-*jun*, and KC genes.** The well-characterized immediate-early genes c-*fos*, NGFI-A, and c-*jun* are inducible in murine fibroblasts by the activation of both receptor PTKs and PKC (28, 35, 45,

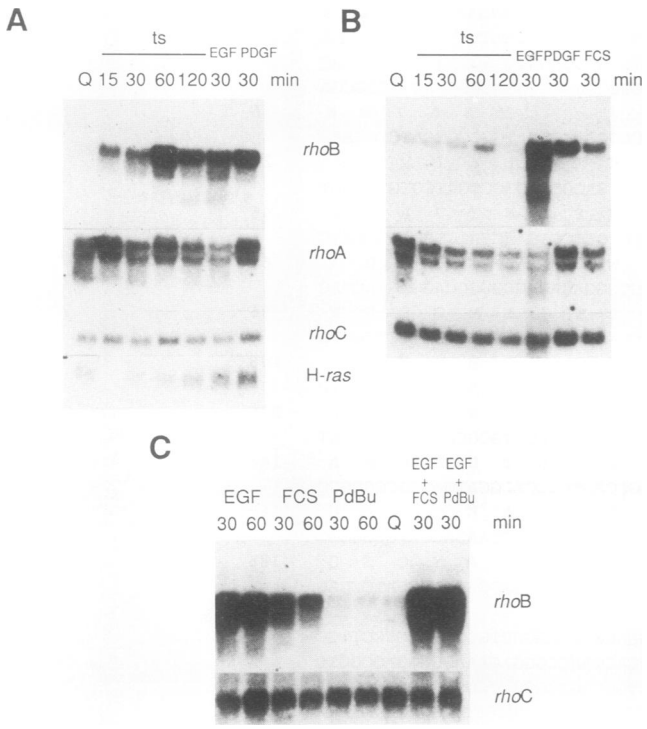

FIG. 2. Induction of *rhoB* but not of *rhoC*, *rhoA*, and H-*ras* RNA by growth factors and by the v-*fps* oncoprotein. Shown are Northern blots of total RNA from 2491 cells, a Rat-2 clone carrying a *ts* mutant of the v-*fps* oncoprotein (A), from Rat-2 cells (B), and from NRK cells (C). Cells had been rendered quiescent (Q) by serum starvation at 39.5°C in 0.2% FCS, and RNA was prepared at the indicated times either after temperature shift (ts) to 34°C or after addition of the indicated mitogens at 39.5°C. The individual probes were used sequentially with intermediate stripping; the c-H-*ras* probe was used on a parallel membrane loaded with identical samples.

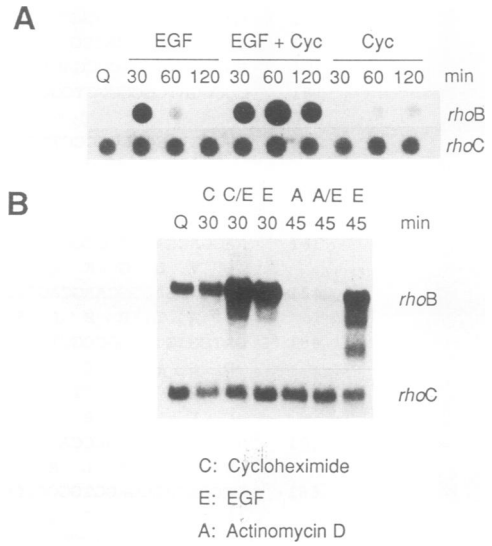

FIG. 3. Induction of *rhoB* by EGF in the presence of cycloheximide but not of actinomycin D. (A) RNA dot blot analysis of total RNA from Rat-2 cells rendered quiescent (Q) by density arrest as described in Materials and Methods. RNAs were isolated at the indicated times after treatment with EGF, cycloheximide (Cyc), or EGF plus cycloheximide. Identical samples were analyzed on membranes prepared in parallel by using the *rhoB* and *rhoC* probes. (B) Northern blot analysis of total RNA from Rat-2 cells rendered quiescent (Q) by serum starvation in 0.2% FCS as described in Materials and Methods. RNAs were isolated at the indicated times after addition of cycloheximide (C), EGF (E), or actinomycin D (A) or of cycloheximide plus EGF. The EGF treatment in the presence of actinomycin D lasted 35 min, following a pretreatment with actinomycin D for 10 min.

52, 56, 57). In EGF-treated Rat-2 cells, these genes are transcriptionally activated in parallel with the *rhoB* gene (Fig. 4A and B). However, no activation of *rhoB* by phorbol esters had been found in the same cells (Fig. 2). To characterize further the regulation of *rhoB* by comparison with other immediate-early genes, we analyzed their induction by mitogens and by the v-*fps* PTK both in serum-deprived and in density-arrested Rat-2 cells. Using Northern and dot blot procedures, we estimated the amounts of cellular RNA present in each sample by comparison with the noninducible *rhoC* and *rhoA* genes (see above). A 30-min treatment with PdBu led to an induction of the c-*fos* RNA from undetectable levels by a concentration as low as 0.8 ng/ml (Fig. 5A). As shown before in murine fibroblasts, this response was more pronounced in density-arrested cells (53). Nearly identical results were obtained for the regulation of the NGFI-A gene (data not shown). In contrast, the expression of *rhoB* was easily detectable in resting cells, being more abundant in serum-deprived than in density-arrested cells. However, no induction of *rhoB* RNA occurred within 30 min after treatments with concentrations of PdBu up to 500 ng/ml under either condition of cell quiescence (Fig. 5A) or after stimulation with 200 ng of PdBu per ml for up to 2 h (data not shown).

The dose-response analysis of Rat-2 cells stimulated by FCS revealed further differences in the regulation of *rhoB* and c-*fos*. Under conditions of serum deprivation, c-*fos* was reasonably strongly induced by the addition of FCS to the medium at a final concentration of 0.6% (Fig. 5B). In contrast, 4% FCS was required for a minimal induction of *rhoB*. These differences were more pronounced under conditions of density arrest, when 10% FCS did not induce *rhoB* (Fig. 5C). In contrast, the EGF dose-response curves of c-*fos* and *rhoB* under both conditions of cell quiescence were nearly identical (Fig. 5B and C). Similarly, no significant differences in the regulation of these genes by EGF and PDGF or differences in the inducibility of c-*fos* and NGFI-A by EGF, PDGF, and FCS were apparent (data not shown). c-*jun* is inducible by TPA in Rat-2 cells (42), but neither KC nor *rhoB* was inducible by TPA in Rat-2 cells (data on KC not shown). Both genes were inducible by FCS (Fig. 5), but unlike *rhoB*, KC was not inducible by EGF. A pattern of gene induction similar to that in density-arrested Rat-2 cells was observed in density-arrested 2491 cells (Fig. 6). As for Rat-2 cells, *rhoB* was not inducible by PdBu or 10% FCS, but *rhoB*, as well as NGFI-A and c-*fos*, was inducible relative to *rhoA* by shift to the permissive temperature as well as by EGF treatment.

DISCUSSION

To characterize the nuclear consequences of nonreceptor PTK activity, several groups including ours have isolated cDNAs of RNAs inducible by a *ts* v-*src* PTK (5, 32, 63, 65). In this study, we have shown that one of these cDNA clones originated from the *rhoB* gene of the rat and have studied its

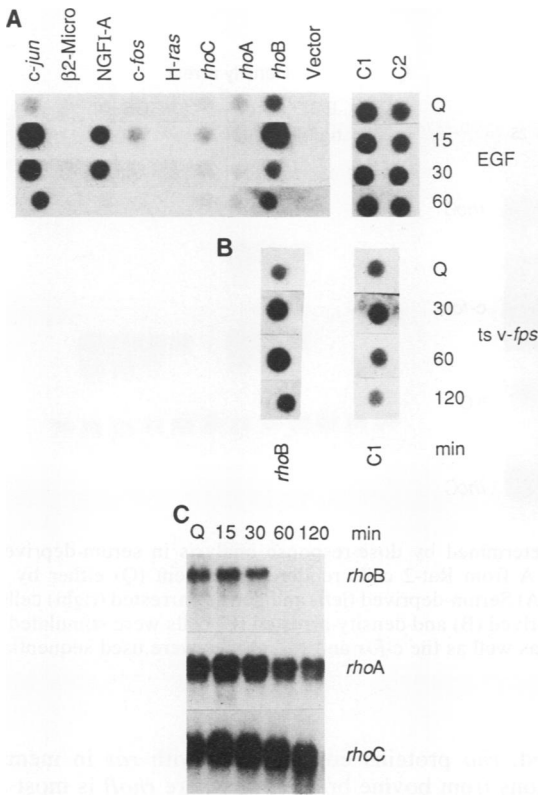

FIG. 4. Transcriptional activation of *rhoB* by EGF and by the v-*fps* oncoprotein. (A and B) Dot blots of cDNA plasmids hybridized to ^{32}P-labeled nuclear run-on transcripts from Rat-2 or 2491 cells as described in Materials and Methods. Nuclei were prepared from cells rendered quiescent (Q) by density arrest at 39.5°C and at the indicated times after stimulation of Rat-2 cells with EGF at 39.5°C (A) or after temperature shift of 2491 cells to 34°C (B). C1 and C2, rat cDNAs of unknown sequence corresponding to constitutively expressed RNAs (see Materials and Methods); β2-Micro, β_2-microglobulin. The hybridization signals do not reflect the relative abundances of mRNAs, since the lengths of the cDNA inserts vary to a large extent (see Materials and Methods). (C) Northern blot analysis of total RNA from Rat-2 cells rendered quiescent (Q) by serum starvation at 37°C in 0.2% FCS as described in Materials and Methods. Individual plates were treated with actinomycin D, and RNA was isolated at the indicated times. The probes were used sequentially on the same membrane.

regulation. Its RNA is labile, with a half-life of less than 30 min. Upon activation of a *ts* v-*fps* PTK or after EGF treatment of Rat-2 fibroblasts, *rhoB* was rapidly and transiently induced at the transcriptional level. In the presence of cycloheximide, its induction was enhanced and prolonged. Such properties are typical for a group of mitogen-inducible, so-called immediate-early genes (1, 36, 37). Three stretches of A+T-rich sequences including ATTTA motifs are present in the 3′ untranslated sequence of the *rhoB* cDNA. Such sequences have been implicated in the rapid degradation of labile RNAs (61), and they may account for the short half-life of *rhoB* mRNA.

The maximal response to the v-*fps* PTK was delayed by 15 to 30 min relative to the induction by EGF treatment. However, an increase of *rhoB* expression was detectable within 15 min after temperature shift, indicating that the induction by the v-*fps* PTK is a direct effect and that the delay results from transport and refolding of inactive v-*fps* molecules that are inappropriately localized at the nonpermissive temperature. No response of the *rhoB* gene occurred after treatment with other activators of receptor PTKs such as insulin or bFGF. In Rat-2 and NRK cells, the c-*fos* and NGFI-A genes are less well stimulated by these agents than by EGF and PDGF. Similar results have been reported for the FGF response of a group of immediate-early genes in mouse fibroblasts (37). These effects may not be specific for *rhoB* and may rather reflect low receptor numbers or a lack in the intracellular response mechanisms to these receptors in such cells.

Particularly noteworthy is the lack of response of *rhoB* to activation of PKC. Other immediate-early genes such as c-*fos* and NGFI-A were inducible through this pathway in the same experiments, excluding cell line-specific effects. All other immediate-early genes that have been analyzed in mouse fibroblasts are well induced by phorbol esters, serum, and PDGF (28, 35–37, 52, 56, 58). The weak response of *rhoB* to FCS treatment, particularly in cells rendered quiescent by density arrest, further emphasizes the unusual regulation of *rhoB*. The SRE and TRE are the two promoter sequence elements of immediate-early genes that have been identified as targets for both the receptor PTK- and PKC-mediated signal transduction pathways. Thus *rhoB* may be regulated by a distinct promoter element that is responsive to signals from retroviral and receptor PTKs but not to those from PKC. This element seems to be different from the FCS-responsive element(s) of KC, which does not respond to phorbol ester treatment since KC is not EGF inducible in Rat-2 cells.

Other genes directly inducible by v-*src* or v-*fps* include 9E3 or CEF4 (5, 65), which encode a secreted peptide related to a family of mitogenic and chemotactic factors, CEF10 (63), a glucose transporter gene (6), and c-*fos* and NGFI-A, which both encode DNA-binding proteins (32). All of these genes are inducible by serum, indicating that many of the more than 80 known serum-inducible genes may be inducible by retroviral PTKs as well. Although none of the regulatory elements in such genes has been identified, the SRE of c-*fos* has been identified as one of the targets for the signals derived from the v-*src* PTK, and in v-*src*-transformed cells, the activities of the DNA-binding proteins AP-1 and PEA3 and the CCAAT-binding protein are increased. An analysis of the *rhoB* promoter region will be needed to determine what elements and factors are involved in its induction by different PTKs.

There are numerous examples of genes that are induced directly or indirectly by the v-*src* and v-*fps* PTKs (5, 6, 32, 63–65). Although none of these on its own may elicit the fully transformed state of a fibroblastic cell, antisense ablation and antibody microinjection experiments indicate that the induction of some of the immediate-early genes that act intracellularly is necessary for resting cells to proceed through the G_1 phase of the cell cycle. It will be important to test whether there is a requirement for *rhoB* expression for progression through G_1.

rhoA, *rhoB*, and *rhoC*, also known as *rho12*, *rho6*, and *rho9*, respectively (13), are members of the *ras* superfamily of low-molecular-weight GTP-binding proteins of ~20 to 30 kDa that possess conserved GTP-binding and hydrolysis domains. The precise cellular functions of these proteins are unknown, but the structural relationship to the heterotrimeric G proteins and to elongation factor Tu suggests roles in signal transduction or in mediating a variety of intracellular interactions, including organelle traffic (for a review, see reference 29). The precise intracellular localization of the

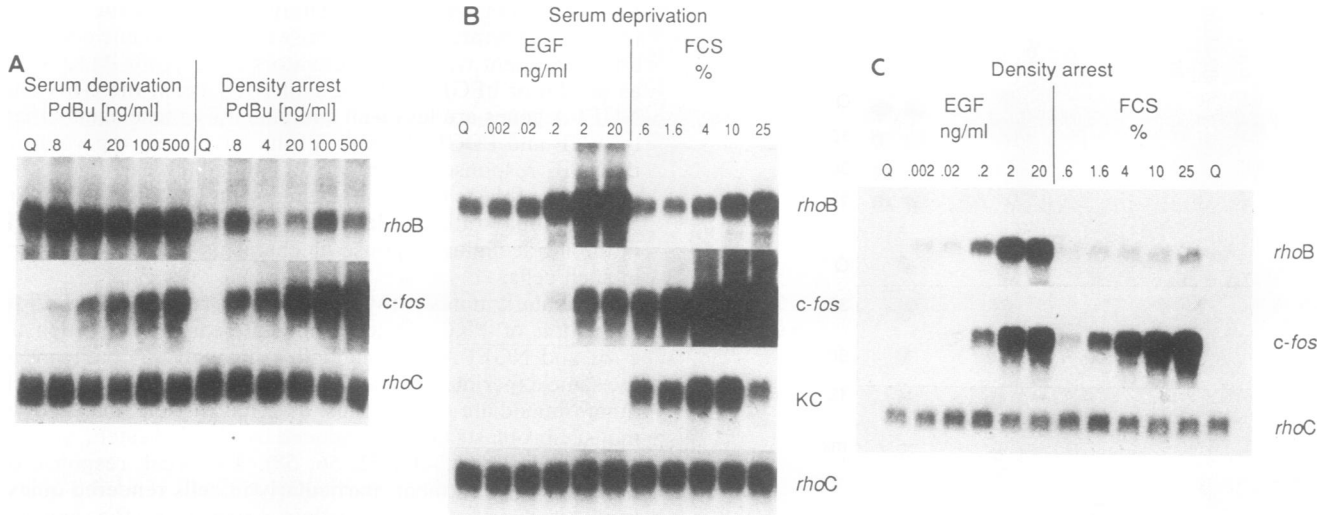

FIG. 5. Differential regulation of *rhoB*, c-*fos*, and KC by mitogens as determined by dose-response analysis in serum-deprived and density-arrested Rat-2 cells. Shown is a Northern blot analysis of total RNA from Rat-2 cells rendered quiescent (Q) either by serum starvation for 20 h or by density arrest as described in Materials and Methods. (A) Serum-deprived (left) and density-arrested (right) cells were stimulated for 30 min at the indicated final concentrations of PdBu. Serum-deprived (B) and density-arrested (C) cells were stimulated for 30 min at the indicated final concentrations of EGF or FCS. The *rhoB* and *rhoC* as well as the c-*fos* and KC probes were used sequentially on two membranes prepared from identical samples.

rho proteins is not known. However, they share with the *ras* proteins a C-terminal sequence that is subject to several posttranslational changes resulting in cleavage, lipid modification, and membrane anchoring (30). Interestingly, *rhoB* differs from *rhoA* and *rhoC* in having additional cysteines upstream of the cysteine in the CAAX box that is modified by isoprenylation. In *ras*, one or more of these upstream cysteines is modified by palmitoylation. Instead, *rhoA* and *rhoC* have a basic region upstream of the CAAX box, similar to that in c-K-*ras* type 4B, which has been implicated in membrane association. Thus, the *rhoB* protein, like the *ras* proteins, may be localized to plasma membranes and may have a localization distinct from those of *rhoA* and *rhoC*.

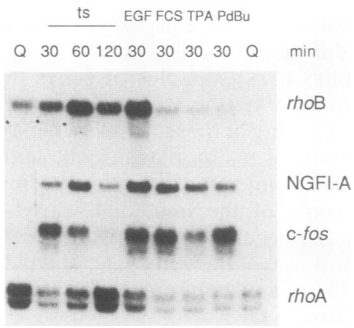

FIG. 6. Coordinate response of *rhoB*, NGFI-A, and c-*fos* to the v-*fps* oncoprotein and to EGF but not to FCS or to phorbol esters under conditions of density arrest. Shown is a Northern blot analysis of total RNA from 2491 cells rendered quiescent (Q) by density arrest as described in Materials and Methods. The cells were stimulated for the indicated times either by temperature shift (ts) to 34°C or by the addition of EGF, FCS, or the phorbol ester TPA or PdBu. Parallel blots prepared from identical samples were analyzed sequentially either with the *rhoB* and *rhoA* probes or with the NGFI-A and c-*fos* probes. *rhoA* served to normalize for the uneven loading of RNAs in different lanes.

Indeed, *rho* proteins cofractionate with *ras* in membrane fractions from bovine brain (34), where *rhoB* is most abundant.

To our knowledge, *rhoB* is the only immediate-early gene among the *ras* family. In contrast to *rhoB*, *rhoA* and *rhoC* are not affected by mitogens or by the v-*fps* PTK. No distinct functions of the individual members of these *ras*-like proteins are known, although as discussed above, their C-terminal sequences are divergent and evolutionarily conserved. The selective induction of *rhoB* may indicate that the *rhoB* protein plays an opposing role to the *rhoA* and *rhoC* proteins in cell physiology. Interestingly, strong overexpression of *rhoA* results in a mildly transformed phenotype in rat and murine fibroblast lines (3).

Both EGF and the v-*fps* PTK induce *rhoB* RNA transiently. Depending on the half-life and pool size of the *rhoB* protein, this may result in a transient or a prolonged increase of *rhoB* protein during G_1. The *rho* and *rac* proteins are in vitro substrates of the botulinum toxin C3 enzyme (12, 22). Microinjection of a mutationally activated *rhoA* protein or C3 induces changes in cell morphology (12, 49). C3 causes a loss of microfilaments (12, 49), whereas the activated Val-14 mutant *rhoA* causes a dramatic increase in microfilaments when injected into quiescent mouse fibroblasts (49). These results suggest that the *rho* proteins may play a role in reorganization of the cell shape, which occurs during mitogenesis and transformation (7, 8), through regulation of cytoskeletal organization. The *ras* proteins bind the GTPase activator protein GAP120, which is phosphorylated by receptor PTKs and the v-*src* and v-*fps* PTKs (23, 44). Perhaps the analogous *rho* GAP (26), which is distinct from GAP120, is subject to similar modifications, which would provide another connection between *rho* and the PTK pathway. The first step in elucidating a possible role for *rho* mitogenesis will be to determine to what extent the abundance of *rhoB* protein changes in response to induction of its mRNA and how fast this change is in comparison with the changes in cell morphology.

Expression of both the *rho* and *ras* genes occurs in a variety of tissues (47) and thus is probably not restricted to fibroblasts. The inducibility of the *rhoB* RNA under conditions of high cell density may indicate a role for *rhoB* in PDGF-, EGF-, and TGF-α-stimulated cellular growth during wound repair, tissue regeneration, embryogenesis, and tumorigenesis.

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