Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 12549–12553, December 1994
Genetics

Genetic selection of growth-inhibitory sequences in mammalian cells

(mitogen-activated protein kinase/c-Fos/JunB/selectable expression of transient growth arrest phenotype/growth control)

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Communicated by Daniel Nathans, August 24, 1994 (received for review July 9, 1994)

ABSTRACT **To assess the role of mitogenically activated genes in the control of cell proliferation, we have taken a genetic approach based on the premise that blocking the function of an essential gene should lead to growth inhibition. Using a newly developed selection procedure, we isolated growth-inhibitory sequences from a pool of random cDNA fragments of 19 growth-related genes associated with the G_0/G_1 transition. These sequences encode potential dominant negative variants of c-Fos, JunB, and $p44^{MAPK}$ that may interfere with their growth-related functions. We anticipate that this procedure, which allows for the selection of sequences that cause a growth-inhibition phenotype, may have broad applications in the identification and analysis of genes that regulate cell growth.**

Efforts to understand the genetic control of mammalian cell growth include the identification of oncogenes and tumor suppressor genes, genes expressed at specific phases of the cell cycle, and genes whose products have growth-regulated activities. In one approach, several laboratories have identified nearly 100 genes induced during the G_0/G_1 transition by differential hybridization screening of cDNA libraries (1–4). Many of these genes are termed immediate-early genes, as their expression is rapidly activated by mitogens without requiring *de novo* protein synthesis. Although this expression pattern suggests that these genes may play important roles in regulating growth, much of the evidence for their requirement in cell proliferation is circumstantial (5, 6).

We sought to develop a strategy to provide evidence that the function of a gene product is required for cell proliferation. If a gene is essential for cell proliferation, one may expect that disruption of its function should lead to growth arrest. Based on this concept, we have devised a genetic selection procedure to isolate growth-inhibitory sequences in mammalian cells. Using this procedure, we show that sequences encoding fragments of c-Fos, JunB, and $p44^{MAPK}$ inhibit growth of murine fibroblasts when expressed. These results indicate that proteins of the AP1 and mitogen-activated protein (MAP) kinase families play essential roles in the growth of fibroblasts. This selection procedure may be useful in identifying other growth-inhibitory sequences, including genes that might regulate cellular quiescence and senescence.

MATERIALS AND METHODS

Cell Culture. All cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (HyClone) and penicillin-streptomycin (GIBCO). The single-cell cloned LAP3 line was derived from NIH 3T3 cells cotransfected (7) with pHβALAP267 (8) and pSV2-neo (9) after G418 selection. To induce expression of transfected sequences, isopropyl β-D-thiogalactopyranoside (IPTG) (dioxane-free; Sigma) was added to the medium to 1 mM.

Construction of Inducible Expression Vectors. pX8B6 and pX11 (see Fig. 2*A*) were constructed by standard techniques. The *Sac* I/*Pvu* II fragment containing an *Escherichia coli* origin of replication, ampicillin-resistance gene, and f1 origin were derived from a *Fsp* I/*Pvu* II fragment of pBluescript SK− (Stratagene). The promoter sequence was from the 0.4-kb *Sac* I/*Sma* I fragment of pMAMneo (Clontech), and the simian virus 40 polyadenylylation signal was from pSG5 (10). Fifteen tandemly repeated copies of the *lac* operator sequence were obtained by partial digestion of pL21CAT (8) with *Sal* I and *Pvu* II. Appropriate oligonucleotides were used to create the multiple cloning site.

BrdUrd/Light Treatment. Cells (1×10^5) were seeded in a 10-cm dish, and BrdUrd (Sigma) was added to the medium to 125 μM. The medium containing BrdUrd was changed once 36 hr later. After the cells were exposed to BrdUrd for 60 hr, the medium was replaced with DMEM containing Hoechst 33258 (4 μg/ml) (Sigma). Three hours later, dishes were placed on a sheet of clear glass 5 cm directly above a 15 W fluorescent bulb (daylight type, Sylvania Electric Products, Fall River, MA), covered with aluminum foil, and irradiated from beneath for 15 min. Cells were then washed once with phosphate-buffered saline and fresh DMEM was added. To facilitate the detachment of dead cells, the dishes were treated with trypsin 3–14 hr after irradiation and the cells were left to grow in the same dish. The medium was subsequently changed on days 2 and 5. Virtually 100% of quiescent LAP3 cells survived this treatment, while logarithmically growing cells were efficiently killed with a background of $<10^{-5}$. The BrdUrd/light procedure as described here works well provided that cells are seeded at a sufficiently low initial density to avoid clustering.

Construction of Random Fragment Library. A random fragment library was prepared by using coding sequences of the cDNA clones of 19 genes, including the following: c-*fos* (11), c-*jun* (12), *junB* (13), *junD* (14), c-*myc* (15), *SRF* (16), *nur77* (17), *hlh462* (18), *nup475* (19), *zif268* (20), *MPK-1* (21), *erk1* (22), *erk2* (23), $pp90^{rsk}$ (24), $pp70^{S6K}$ (25), *odc* (26), *cyr61* (27), *gly96* (28), and *pip92* (29). The cDNAs of these genes were separated from the vector sequences by agarose gel electrophoresis. Equal amounts of each purified insert were mixed and used as templates for a random priming reaction. Approximately 50 ng of the mixed template was boiled with 1 pmol of the $A2/N_6$ primer 5′-CCTAGCTACCATGGN_6-3′ for 3 min, cooled on ice, mixed with a reaction mixture containing 10 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.125 mM each dNTP, and 2.5 units of Klenow fragment of DNA polymerase and incubated at 37°C for 15

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Abbreviations: MAP kinase, mitogen-activated protein kinase; IPTG, isopropyl β-D-thiogalactopyranoside; SETGAP, selectable expression of transient growth arrest phenotype; GSE, genetic suppressor element.

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min. Then the reaction mixture was boiled and cooled again, another 2.5 units of Klenow fragment was added, and the reaction mixture was incubated at 37°C for 15 min. The reaction was stopped by boiling and diluted 1:10 with TE buffer and products were separated from primers using Microcon-100 microconcentrators (Amicon) with two washes of the retained solution with 200 μl of the TE buffer. After two cycles of priming, some reaction products incorporated primer sequences at both ends. They were amplified in PCR using A2/RI primer 5′-AGGAATTCTACCTAGCTACCAT-3′ (underlined sequence overlaps with the 5′ portion of $A2/N_6$). Several micrograms of the amplified products were precipitated, purified with Microcon-100, cut with *Eco*RI, and cloned into the *Eco*RI site of pX8B6. The resulting library was estimated to contain 3×10^5 clones with a size range of 200–1200 bp. The primer sequences were designed to incorporate an ATG codon at the 5′ end of the cloned sequences and stop codons in all three frames at the 3′ end.

Transfection. Five micrograms of the library DNA was cotransfected with 0.5 μg of plasmid pHyg carrying the hygromycin-resistance gene (30) and 10 μg of carrier NIH 3T3 DNA into LAP3 cells (5×10^5 cells per 10-cm dish) by the calcium phosphate method (7). The stably transfected cells were selected in the medium containing hygromycin (120 units/ml) (ICN) for 2 weeks.

Isolation of Library Sequences from Cells After Selection. Total RNA was isolated (31) after a 15-hr exposure of the cells to 1 mM IPTG and used to generate cDNA with an oligo(dT) primer. To amplify specifically library-derived IPTG-induced sequences, PCR of the cDNA was carried out with M5 and P3 primers (see Fig. 2*B*). The amplified products were directionally recloned into pX11 by a procedure designed to avoid possible disruption of the sequences with restriction enzymes. Briefly, the PCR products were reamplified in PCR with the primers 5′-pCTAGCTTGATATCGAATTCTAC-3′ and 5′-pCCGGCTGCAGGAATTCT-3′. The resulting products were treated with *Exo* III to create protruding ends compatible with *Bsp*EI and *Nhe* I restriction sites (32) and cloned into *Bsp*EI and *Nhe* I-cut and dephosphorylated pX11.

Immunoprecipitation. Cells were incubated for 2 days in medium containing 1 mM IPTG for 2 days and then labeled for 3 hr with Tran^{35}S-label (ICN). The fos26 polypeptide was immunoprecipitated using Fos1 monoclonal antibodies (Oncogene Science).

RESULTS

Design of the Selection Procedure. We devised a genetic selection procedure to isolate sequences that inhibit growth in mammalian cells (Fig. 1). This procedure, called SETGAP (selectable expression of transient growth-arrest phenotype), incorporates three components: (*i*) a source of growth-inhibitory sequences; (*ii*) a mammalian-inducible system that permits the on/off expression of transfected sequences; and (*iii*) a positive selection for growth-arrested cells. Potentially growth-inhibitory sequences are cloned in an inducible expression vector and transfected into test cells. These cells are then induced to express the transfected sequences, some of which may cause growth inhibition when expressed, and then subjected to treatment that preferentially kills growing cells. The surviving, growth-inhibited cells are rescued by removal of the inducer. Transfected sequences from them can be isolated and analyzed or used for another round of selection before analysis.

To obtain growth-inhibitory sequences, we used the genetic suppressor element (GSE) approach (33–35) to generate a library of sequences that might be capable of inactivating various growth-related genes. The rationale of this approach is that in a pool of randomly fragmented cDNAs, expression of some of the fragments may inhibit the function of their cognate genes if they encode either an effective antisense RNA or a dominant negative variant of the wild-type protein product of the gene (36). Coupled with an appropriate genetic selection, this procedure should enable identification of sequences from a GSE library that interfere with the activities of a gene product without prior knowledge of its structure or function. Although the SETGAP procedure described here utilizes a GSE library, it could also be used to analyze unfragmented cDNAs. For example, growth inhibition may result from synthesis of proteins that negatively regulate growth or it may be due to a gain of function or the stoichiometric imbalance of components in multiprotein complexes (37, 38).

Establishment of an Inducible Expression System. Since we aim to isolate sequences that cause growth inhibition when expressed, the ability to control their expression is essential to propagate the cells that carry them. A suitable vehicle for such sequences must allow the on/off regulation of transfected sequences with a low level of expression in uninduced cells. We adapted an IPTG-inducible mammalian expression system that utilizes a chimeric protein, LAP267, which in the presence of IPTG activates transcription from a promoter linked to *lac* operator sequences (8). We generated an NIH 3T3 cell-derived cell line, designated LAP3, which expresses the LAP267 transactivator. The original IPTG-inducible vector pL21CAT (8) contains the simian virus 40 early promoter and exhibits significant background expression in LAP3 cells. We thus constructed two IPTG-inducible expression vectors, pX8B6 and pX11 (Fig. 2), which contain *lac* operator sequences upstream of the basal part of the mouse mammary tumor virus promoter. These vectors provide a low level of background expression in LAP3 cells in the absence of IPTG

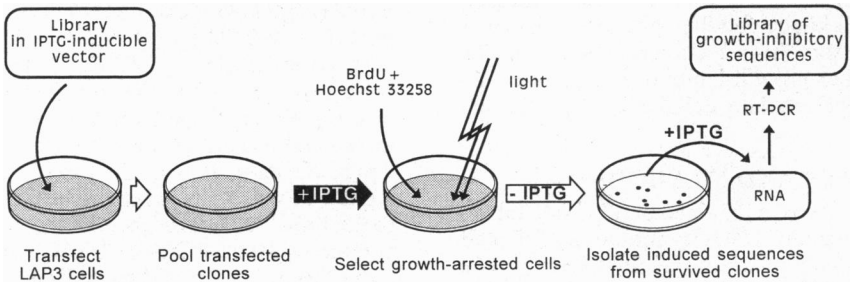

FIG. 1. SETGAP selection for growth-inhibitory sequences in mammalian cells. Potentially growth-inhibitory sequences are cloned in an IPTG-inducible expression vector and transfected into test cells—LAP3 in this study. Stable transfectants are obtained in the absence of IPTG. IPTG is then added and cells that undergo transient growth arrest are selected by combined treatment with BrdUrd, Hoechst 33258, and visible light. Cells synthesizing DNA are killed in this process, and growth-arrested cells can be rescued by IPTG withdrawal. Sequences expressed upon IPTG addition are isolated from surviving cells by reverse transcriptase (RT) PCR using vector-specific primers. These sequences are recloned to obtain a secondary library, with which the selection can be repeated if necessary. Alternatively, individual clones from the library can be isolated and characterized.

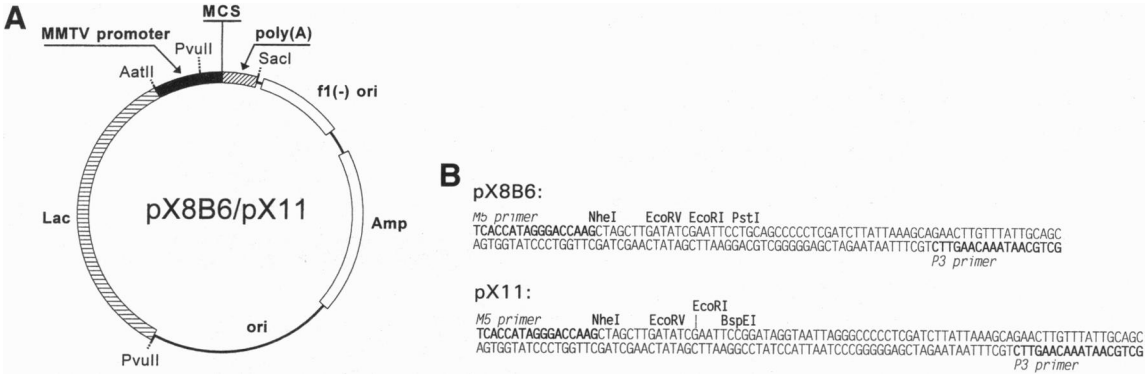

FIG. 2. IPTG-inducible vectors pX8B6 and pX11, which are identical except for their multiple cloning sites (MCS), shown in *B*. Lac, lactose operator sequences (8); MMTV, mouse mammary tumor virus.

(see Fig. 5*A*). In the presence of IPTG, the test gene encoding luciferase was inducible up to 300-fold (data not shown).

Positive Selection for Growth-Inhibited Cells. To select positively growth-inhibited cells, we used a procedure that exploits the fact that proliferating cells incorporating BrdUrd into their DNA are killed by visible light after exposure to the fluorescent dye Hoechst 33258 (39, 40). Growth-arrested cells, which do not synthesize DNA and thus do not incorporate BrdUrd, are not photosensitive and survive this treatment. The BrdUrd/light procedure is highly efficient and allows selection of a few growth-arrested cells among 10^6 growing cells.

Selection of Growth-Inhibitory Sequences. To assess the feasibility of the SETGAP approach, we tested the ability of cDNA fragments of 19 growth-related genes to function as GSEs that inhibit cell growth. Most of these genes are growth factor-inducible immediate-early genes; some encode mitogen-activated kinases (see *Materials and Methods*). Random cDNA fragments of a mixture of these 19 genes were generated from the full-length cDNAs and cloned into pX8B6. The resulting cDNA-fragment library was cotransfected into LAP3 cells along with the plasmid pHyg, which confers resistance to hygromycin.

From several independent transfections, separate pools of ≈500 hygromycin-resistant clones each were obtained; 10^5 cells from each pool were exposed to BrdUrd in the presence of IPTG and then irradiated to kill cells incorporating BrdUrd. Cells rendered unable to grow due to expression of transfected sequences should survive this treatment, and these cells were rescued by growth in the absence of IPTG. After 5 days in its absence, the transfected sequences were isolated from these surviving cells by PCR using primers specific for the expression vector. These sequences, which should be enriched for those that can cause growth arrest when expressed, were recloned into pX11 to produce secondary libraries.

Five such secondary libraries were separately transfected into LAP3 cells; 10^5 cells from each pool were again exposed to BrdUrd in the presence of IPTG and then irradiated. After the BrdUrd/light treatment, surviving cells from each library were allowed to grow for 2 weeks and then split into three dishes. The three dishes corresponding to each library were again treated according to the BrdUrd/light protocol, with two from each set exposed to BrdUrd in the presence of IPTG and a third without IPTG. From one dish of IPTG-treated cells, transfected sequences were recovered and cloned into pX11. Cells in the remaining two dishes from each set, corresponding to the selection with and without IPTG, were grown for 12 days and stained to examine the effect of the IPTG-mediated induction on the number of growth-arrested cells during the BrdUrd/light treatment. Four of five libraries tested showed a clear increase in the number of surviving colonies after the BrdUrd/light treatment in the presence of IPTG (Fig. 3*A*).

Analysis of the library DNA recovered from cells after the first and final rounds of selection showed that its complexity was greatly reduced in the course of selection (Fig. 3*B*). Strikingly, DNA recovered after the final round appeared as a few bands on an agarose gel. Among these DNA species should be those capable of inhibiting cell growth when expressed; we thus tested them individually. The major DNA species (Fig. 3*B*, lanes 3A–3E) were purified from a gel and recloned into pX11; six different clones were thus obtained. Induced expression of three of them caused reversible growth arrest as judged by the BrdUrd/light assay (Fig. 4*A*).

Growth-Inhibitory Sequences. Sequence analysis of the three growth-inhibitory clones revealed that all represent fragments of coding sequences in the sense orientation. These sequences encode protein fragments whose structures suggest that they may act as dominant negative variants of wild-type proteins (Fig. 4*B*). Clones 11-11 and fos26 encode fragments of the JunB and c-Fos proteins, respectively, that

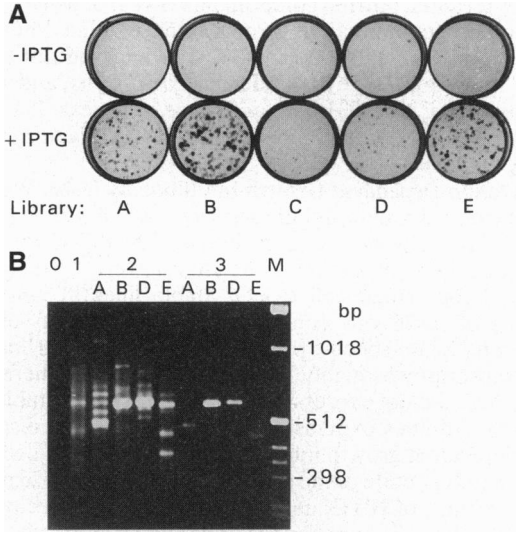

FIG. 3. Analysis of library DNA. (*A*) Cells transfected with secondary libraries, designated A–E, were exposed to BrdUrd, Hoechst 33258, and light in the presence and absence of IPTG. Staining of the dishes reveals colonies derived from cells that were growth arrested in the presence of IPTG and thus survived the BrdUrd/light treatment. (*B*) Agarose gel electrophoresis of the products of PCR amplification of the original, unselected library (lane 1) and library-specific sequences expressed in cells after the first (lanes 2A–2E) and second (lanes 3A–3E) rounds of selection. Lanes 2A–2E represent DNA used to generate secondary libraries shown in *A*. Alphabetical designation of libraries corresponds to those in *A*; lane 0, control PCR (no template); lane M, molecular weight markers—sizes (bp) are indicated on the right.

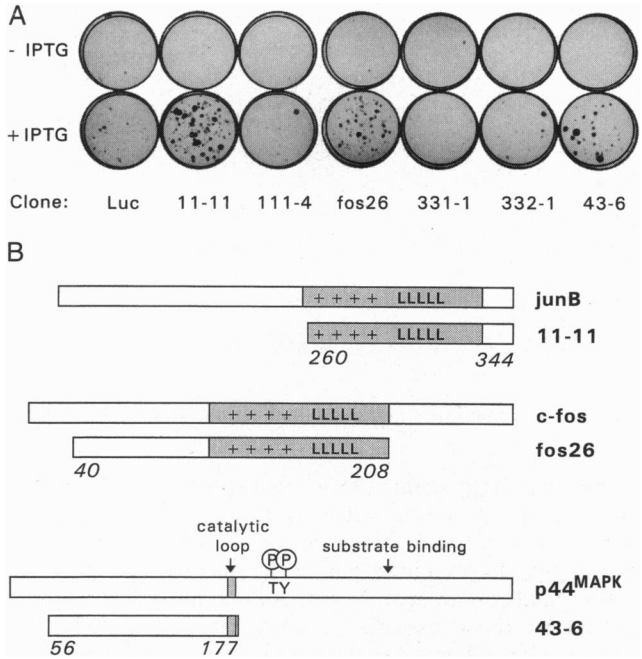

FIG. 4. Analysis of individual clones. (*A*) Six clones obtained after two rounds of selection and a control plasmid (pX8B6-Luc) were transfected into LAP3 cells. Stably transfected cells were pooled and treated according to the BrdUrd/light protocol with or without IPTG. Induction of expression of clones 11-11, fos26, and 43-6 isolated from libraries A, B, and E, respectively (see Fig. 3), resulted in increased survival of cells in the assay. (*B*) Structures of protein products encoded by growth-inhibitory clones. Indicated are first and last amino acid positions. Basic leucine zipper domains are indicated for Fos and Jun polypeptides, and activating phosphorylation sites are shown for $p44^{MAPK}$.

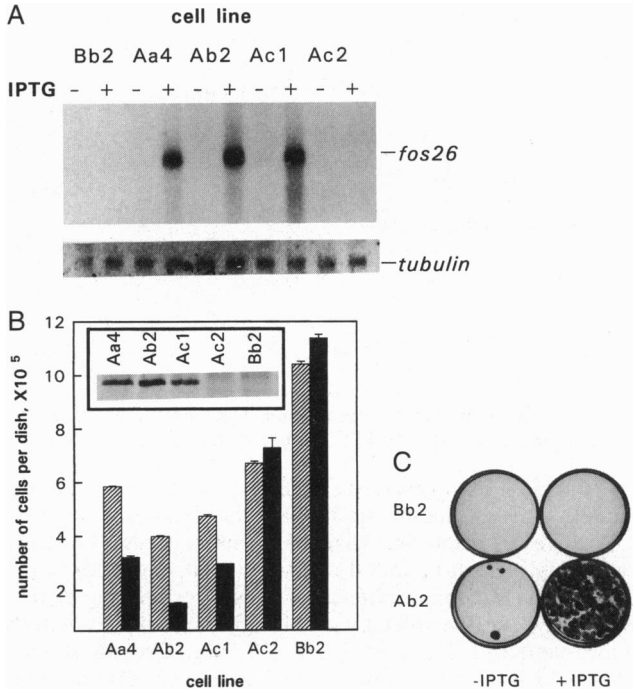

FIG. 5. Growth inhibition by fos26. (*A*) Induction of fos26 transcript in stable cell lines. Stable cell lines Aa4, Ab2, Ac1, and Ac2 represent single-cell clones obtained by transfection of LAP3 cells with pX8B6-fos26. Control cell line Bb2 was similarly obtained with pX8B6-luciferase DNA. Total RNA was isolated from cells after 18 hr in medium with or without IPTG (1 mM) as indicated, separated by electrophoresis, blotted, and probed with labeled fos26 DNA (*Upper*) and α-tubulin (*Lower*). (*B*) IPTG-inducible growth inhibition in cell lines expressing fos26. Cells (5×10^4) were seeded in duplicate in 60-mm dishes and counted 85 hr later after growing in the medium without IPTG (hatched bars) or with 1 mM IPTG (solid bars). (*Inset*) Immunoprecipitation of fos26 polypeptide from lysates of cell lines shown. (*C*) Ab2 clone and control cell line Bb2 were subjected to BrdUrd/light treatment in the presence and absence of IPTG. Growth-arrested cells were rescued by IPTG withdrawal, allowed to grow for 12 days, and stained.

contain the leucine zipper domains. Clone 43-6 encodes part of the N-terminal portion (subdomains II–VI) of $p44^{MAPK}$, the 44-kDa mitogen-activated protein kinase (also known as *erk1*). Clones 111-4, 331-1, and 332-1, which did not lead to growth inhibition, were derived from *cyr61*, c-*fos*, and c-*myc*, respectively. Clone 331-1 is antisense, whereas 111-4 and 332-1 are sense-oriented sequences but do not contain correct reading frames.

Expression-Dependent Growth Inhibition by fos26. We studied one isolated growth-inhibitory sequence, fos26, in greater detail. LAP3 cells were cotransfected with pX8B6-fos26 and pHyg, and hygromycin-resistant clones were selected. We examined four single-cell cloned stable lines in which the presence of fos26 was confirmed by PCR analysis of their DNA with pX8B6-specific primers. In three such cell lines, the fos26 transcript was highly inducible with IPTG, whereas the fourth, Ac2, did not express the transgene at a detectable level (Fig. 5*A*). All lines expressing fos26, but not Ac2, displayed IPTG-dependent growth inhibition (Fig. 5*B*). These cells synthesize a polypeptide of the size expected for the fos26 protein in the presence of IPTG, as detected by immunoprecipitation (Fig. 5*B*). The cells experienced apparent growth arrest upon IPTG treatment but were able to grow and form colonies after removal of IPTG, as evidenced by the BrdUrd/light assay (Fig. 5*C*). Taken together, these data clearly show the expression-dependent inhibition of cell growth by fos26.

DISCUSSION

For over a decade, it has been possible to select and identify gene sequences that positively regulate cell growth through DNA-mediated gene transfer. Many genes that confer a growth advantage, including oncogenes, were thus isolated (41, 42). However, it has not been possible to identify gene sequences that negatively regulate growth based on a growth-inhibition phenotype. In this communication, we report a genetic selection procedure, SETGAP, that allows the selection of growth-inhibitory sequences in mammalian cells. This procedure is based on transfection of cells with sequences cloned under the control of an inducible promoter and the selection of cells that become growth-inhibited when the transfected sequences are expressed. An important feature of this selection scheme is the reversibility of growth inhibition dependent on the presence of an inducer. This feature provides compelling evidence that it is the expression of the isolated sequences that causes the apparent growth arrest.

In this study, we applied the SETGAP procedure as a direct test for the functional requirement of a set of growth-related genes in cell proliferation. Our results provide evidence that proteins of the Jun-Fos family, as well as $p44^{MAPK}$, play essential roles in the growth of murine 3T3 cells. Two of the three growth-inhibitory sequences analyzed encode fragments of c-Fos and JunB proteins that contain the leucine zipper domains. These fragments might dimerize with members of the AP-1 family (43, 44) and sequester them in nonfunctional complexes. These results are consistent with previously reported data that Fos and Jun proteins may be required for the proliferation of cultured fibroblasts (45–48).

Another growth-inhibitory sequence encodes a fragment of $p44^{MAPK}$. The MAP kinase family of proteins plays key roles in signaling pathways after mitogenic stimulation (49, 50). Although the mechanism of growth inhibition by this $p44^{MAPK}$ fragment is not known, it is tempting to speculate

that it might be involved in the interaction of $p44^{MAPK}$ with other proteins, and thus overexpression of this fragment may interfere with the function of $p44^{MAPK}$. A more extensive analysis of GSEs of MAP kinase that inhibit growth may yield insights into structure/function relationships.

The isolated GSEs can serve as reagents in further studies to delineate their corresponding proteins' roles in cell growth. Unlike previous studies in which prior knowledge of the gene structure was required to engineer specific inhibitory constructs, the sequences identified here emerged through genetic selection from a random fragment library. Importantly, however, results of this study do not exclude the possibility that other genes among the 19 tested may be required for growth: first, the current study has not exhaustively examined all clones from the random fragment library; second, some genes may not be efficiently inactivated by GSEs.

The present experimental design allows several relatively simple ways of optimization of the SETGAP procedure for other applications. For example, it should be feasible to increase transfection efficiency and perform additional rounds of selection if one needs to analyze a larger number of sequences or screen a complex library. Alternatively, reducing the complexity of the libraries to be tested may prove useful. The complexity of GSE libraries could be kept in check by reducing the number of cDNAs represented and the extent of cDNA fragmentation. For cDNA libraries, complexity can be reduced substantially by subtraction and normalization (51, 52). With the transfection protocol used here, each transfected cell incorporates multiple plasmid sequences and may effectively serve as a test tube for a pool of clones from the library. Thus, the actual number of sequences analyzed may be significantly larger than the number of transfectants.

This procedure for the selection of growth-inhibitory sequences may have broad applications. First, coupled with the GSE approach, it can be used to analyze extensively any single gene required for growth. Using random cDNA fragments of a gene of interest, it should be possible to identify which portions of the protein might act as dominant negative mutants and thus gain insight into structure/function relationships. This selection may also identify which fragment of the sequence might work effectively when expressed as antisense RNA. Second, this approach could be used to identify previously unknown genes essential for growth. For example, cDNA libraries representing any phase of the cell cycle may be made into GSE libraries and tested in this selection procedure. Finally, this procedure could be a useful tool to screen full-length cDNA libraries to identify previously unknown antiproliferative genes, including growth suppressor genes and genes that might regulate cellular quiescence and senescence.

We thank M. A. Labow and I. J. Davis for generous gifts of reagents and our colleagues for critical readings of the manuscript. This work was supported by funds from the Pew Scholars Program. L.F.L. is an Established Investigator of the American Heart Association.

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