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Cell Transformation by c-*fos* Requires an Extended Period of Expression and Is Independent of the Cell Cycle

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The proto-oncogene transcription factors Fos and Jun form a heterodimeric complex that binds to DNA and regulates expression of specific target genes. Continuous expression of c-*fos* causes transformation of cultured fibroblasts and induces osteogenic sarcoma in mice. To investigate the molecular basis of *fos*-mediated oncogenesis, we developed a conditional cell transformation system in which Fos expression was regulated by isopropyl-β-D-thiogalactopyranoside (IPTG). Synthesis or repression of Fos in L1-3c-*fos* cells occurred rapidly, within 30 min, after the removal or addition of IPTG to the culture medium. However, there was a significant delay between the induction of Fos expression and the appearance of morphological transformation. No effect was observed after 12 h of Fos expression, partial transformation was detected after 24 h, and full transformation required approximately 3 days of continuous Fos expression. Similarly, the transformed cell morphology persisted for at least 2 days after repression of Fos, and a normal phenotype was observed only after 3 days. Fos-Jun complexes, capable of binding to AP-1 sequences, were present continuously during the delay in morphological transformation. Furthermore, increased expression of several candidate Fos target genes, including those encoding Fra-1, transin (stromelysin), collagenase, and ornithine decarboxylase, was detected shortly after Fos induction. The induction of morphological transformation was not dependent on the cell cycle, as it occurred in both cycling and noncycling cells. Thus, the Fos-Jun complexes present before L1-3c-*fos* cells become fully transformed are transcriptionally active. These complexes disappeared, and the Fos target genes were repressed at least 2 days prior to reversion. Our results suggest that cell transformation by Fos requires increased expression of a target gene(s) with a long-lived product(s) that must reach a critical level.

Proto-oncogenes encode key components of the signal transduction networks that regulate cell growth and differentiation. They function at multiple levels, from the plasma membrane to the nucleus, as extracellular growth factors, cell surface receptors, G proteins, protein kinases, and transcription factors (67). Inappropriate expression or mutation of proto-oncogenes can result in abnormal signal transduction leading to oncogenesis. Therefore, a great deal of effort has been devoted to the analysis of proto-oncogene function in the hope that it will reveal information about the molecular mechanisms that govern the growth of normal and tumor cells.

The inducible proto-oncogene transcription factors Fos and Jun are generally believed to lie at the distal end of a signal transduction cascade. They have been proposed to act as nuclear third-messenger molecules that function in coupling short-term signals elicited by extracellular stimuli to long-term adaptive changes in cell phenotype by regulating the expression of specific target genes (12, 55). Fos and several related proteins (Fra-1, Fra-2, FosB, and ΔFosB), although incapable of forming homodimers, can participate in heterodimeric complexes with members of the Jun and ATF/CREB families (18). Dimerization occurs through a coiled-coil structure termed the leucine zipper and results in juxtaposition of basic regions in each protein, located immediately N terminal of the leucine zipper, that form a bipartite DNA-binding domain (27, 40, 45, 82). Protein dimers bind to transcription control elements known as the activator protein 1 (AP-1) and cyclic AMP-responsive element sites (18). Cross-dimerization among members of this superfamily generates a large number of protein dimers with distinct DNA-binding and transcriptional properties. Since c-*fos* is induced in many different cell types by a variety of signals associated with cell proliferation, differentiation, neuronal excitation, and cell death (55), it is likely that it regulates distinct target genes in each of these circumstances. Thus, the biological effect of Fos expression may be dictated by its specific cellular context.

The *fos* oncogene (v-*fos*) was originally identified as the transforming gene responsible for the induction of osteogenic sarcoma by the Finkel-Biskis-Jinkins (FBJ) murine sarcoma virus (14, 15, 21). Subsequently, it was also identified in two independently isolated retroviruses, FBR murine sarcoma virus (17) and the chicken sarcoma virus NK24 (61). Oncogenic activation of *fos* is largely a consequence of deregulation of expression (54). The c-*fos* gene, when expressed continuously at high levels, transforms fibroblasts in culture and induces bone tumors in transgenic mice (29, 53, 54). Structural and functional analyses have revealed that both the dimerization and the DNA-binding functions of Fos are required for cell transformation (37, 59, 74, 91). A small region of Fos spanning approximately 100 amino acids has been shown to be sufficient for transformation of avian and murine cells, although the transformed cell phenotype was not as profound as that obtained with full-length Fos (74, 91). However, a recent report suggests that a C-terminal activation domain is required for the transforming ability of several Fos proteins (86). In any event, in transformed cells, Fos exists primarily in the form of a heterodimeric complex with c-Jun (13, 23). Therefore, it is likely that cell transformation by *fos* is a consequence of the

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combined effects of transcription activation domains present in both Fos and Jun. Although several potential target genes of *fos* have been identified, the target genes responsible for cell transformation are not yet known.

Here we developed a conditional cell transformation system for Fos based on the LacI activator protein (LAP) expression system (44) as an initial step in an attempt to identify and isolate the target genes responsible for the induction of cell transformation. Fos expression was rapidly activated or repressed by removal or addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture medium. However, there was a delay between Fos expression and the induction of morphological transformation. During this lag period, there was a rapid formation of transcriptionally active Fos-Jun complexes accompanied by changes in expression of several Fos target genes. Transformation was not dependent on cell proliferation, and Fos expression by itself was not sufficient to stimulate DNA synthesis or cell cycle progression. Furthermore, Fos expression had no significant effect on the rate of transition from G_0 to S after serum stimulation. These results suggest that Fos does not transform cells by direct regulation of a small number of target genes involved in cell cycle progression. It is possible that a critical concentration of a target gene product(s) must be achieved for cell transformation. The delay incurred in reversion to a normal phenotype implies that this protein(s) must be relatively long-lived.

MATERIALS AND METHODS

Cell culture. Cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (FBS; GIBCO), 2 mM L-glutamine, and antibiotics (100 U of penicillin and 100 μg of streptomycin per ml). IPTG (dioxane free; Sigma) was prepared in water as a 1 M stock solution, sterilized by filtration, and stored at −20°C. For serum stimulation, subconfluent cell cultures were rendered quiescent in 0.5% FBS for 48 h before treatment with 20% dialyzed FBS for an appropriate period.

For growth rate analysis, cells were seeded in six-well plates at a density of 2×10^5 cells per well and propagated in 10 or 0.5% FBS in the absence or presence of IPTG. When growth was monitored under low-serum conditions, cells were initially plated in 10% FBS overnight and then switched to 0.5% FBS for subsequent studies. The cell medium was changed every 2 days. Duplicate cultures were trypsinized in 0.5 ml of trypsin solution and diluted in an equal volume of trypan blue solution (0.4%; Sigma). Viable cells were counted daily in a hemacytometer counting chamber. Doubling times were calculated by using the equation $Y = Y_0(e^{kt})$, where Y is the number of cells on a given day, Y_0 is the number of cells on day 0, t is the time in days, and k is the calculated rate constant; doubling time = $(0.693/k) \times 24$ h.

Plasmids. Plasmids pHβA348 (also referred to as pLAP348) and pL1-3CAT have been described previously (44). pL17*neo* was a gift from M. Labow. The mouse c-*fos* gene was isolated as a 4.7-kb *Hin*dIII-*Bam*HI fragment by digestion of pc-*fos* (mouse)-3 (54) with *Hin*dIII, *Bam*HI, and *Ase*I. The fragment was cloned into pGEM3Z to generate pGEM3Z(H/B)c-*fos* (mouse). A 2.5-kb *Acc*I c-*fos* fragment containing the entire c-*fos* gene with 42 and 100 bp of the 5′ and 3′ untranslated sequences, respectively, was excised from pGEM3Z(H/B)c-*fos*. This 2.5-kb c-*fos* gene was treated with T4 DNA polymerase and deoxynucleoside triphosphates to generate blunt ends and then cloned between the *Stu*I and *Msc*I sites of the L1-3 vector or into the *Eco*RV site of the pCB6$^+$ cytomegalovirus (CMV) vector (63), generating pL1-3c-*fos* and pCMVc-*fos*, respectively.

DNA transfection and generation of stable cell lines. Plasmids pLAP348 (4 μg), pL17*neo* (0.5 μg), and pL1-3c-*fos* (0.5 μg) together or pCMVc-*fos* (0.5 μg) alone was introduced into subconfluent 208F cells by the $CaPO_4$ precipitation method (8). G418 selection (0.4 mg/ml) was applied 48 h posttransfection in medium containing 10% FBS and 10^{-7} M dexamethasone (Sigma). Focus formation assays were carried out in 5% FBS and 10^{-7} M dexamethasone. The presence of dexamethasone enhanced the phenotypic contrast between the normal and *fos*-transformed cells, thereby facilitating focus identification and the selection of transformed colonies (11). Foci were fixed in methanol containing 0.33% methylene and 0.11% basic fuchsin and counted under a dissecting microscope. Homogeneous G418-resistant *fos*-transformed cell lines were obtained through multiple rounds of subcloning. Briefly, transformed Neo^r colonies were picked with a sterile pipetman tip from heterogeneous cell populations and transferred into individual wells of 24-well plates. Transformed colonies derived from single-cell populations were trypsinized and plated sparsely in 100-mm dishes for a final round of subcloning. Cell clonings were conducted in the absence of G418 and dexamethasone.

Pulse-labeling and immunoprecipitation. Cells were incubated in 35-mm-diameter dishes in the presence of 1 ml of methionine-free DMEM at 37°C for 30 min. Dialyzed FBS was added simultaneously at 20% when cultures were serum stimulated. [^{35}S]methionine (>1,000 Ci/mmol; Amersham) was added at the final concentration of 0.2 to 0.5 mCi/ml to pulse label cells for 30 min. Cells were washed once with phosphate-buffered saline (PBS) and lysed in situ in 1 ml of radioimmunoprecipitation assay/PEAL buffer (150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% deoxycholic acid, 1% Triton 100, 10 mM Tris-Cl [pH 7.6], 1 mM EDTA, 80 μM phenylmethylsulfonyl fluoride, 2 μg of leupeptin per ml, 0.15 U of aprotinin per ml). The lysates were cleared by ultracentrifugation (150,000 × *g*) at 4°C for 40 min. The supernatants were aliquoted evenly and incubated with different antisera (2 to 5 μl) at 4°C on a rotating platform for 2 h. *Staphylococcus aureus* cells (20 μl) (Calbiochem) were then added, and the samples were incubated for 20 min at 4°C. The Fos and Jun antisera (αFos 2.2 and αJun 2.2) were raised against full-length recombinant Fos and Jun expressed and purified from bacteria (10). Immunocomplexes were precipitated by centrifugation and washed three times with radioimmunoprecipitation assay buffer and resuspended in dissociation buffer (625 mM Tris.Cl [pH 6.8], 2% SDS, 5% β-mercaptoethanol, 0.1% bromophenol blue, 10% glycerol). The samples were boiled for 10 min and then centrifuged for 5 min. An equal amount of radioactivity in each of the samples was analyzed by electrophoresis on SDS–9% polyacrylamide gels. The gels were fixed in a solution of 40% methanol and 7% acetic acid for 30 min and then treated with Enlightening solution (Du Pont) at room temperature for 30 min. The gels were dried and exposed to Kodak X-Omat AR at −80°C for different lengths of time.

Protein half-lives were determined by pulse-chase experiments. Cultures were pulse-labeled for 15 min and washed once with DMEM containing 10% FBS and unlabeled methionine. The cultures were then chased with the medium for different lengths of time. The cells were harvested and analyzed as described above. The amount of radioactivity in protein bands corresponding to different points was determined with a Betagen counter.

[^{3}H]thymidine incorporation assay. [^{3}H]thymidine incorporation was monitored by labeling cells with [*methyl*-^{3}H]thymi-

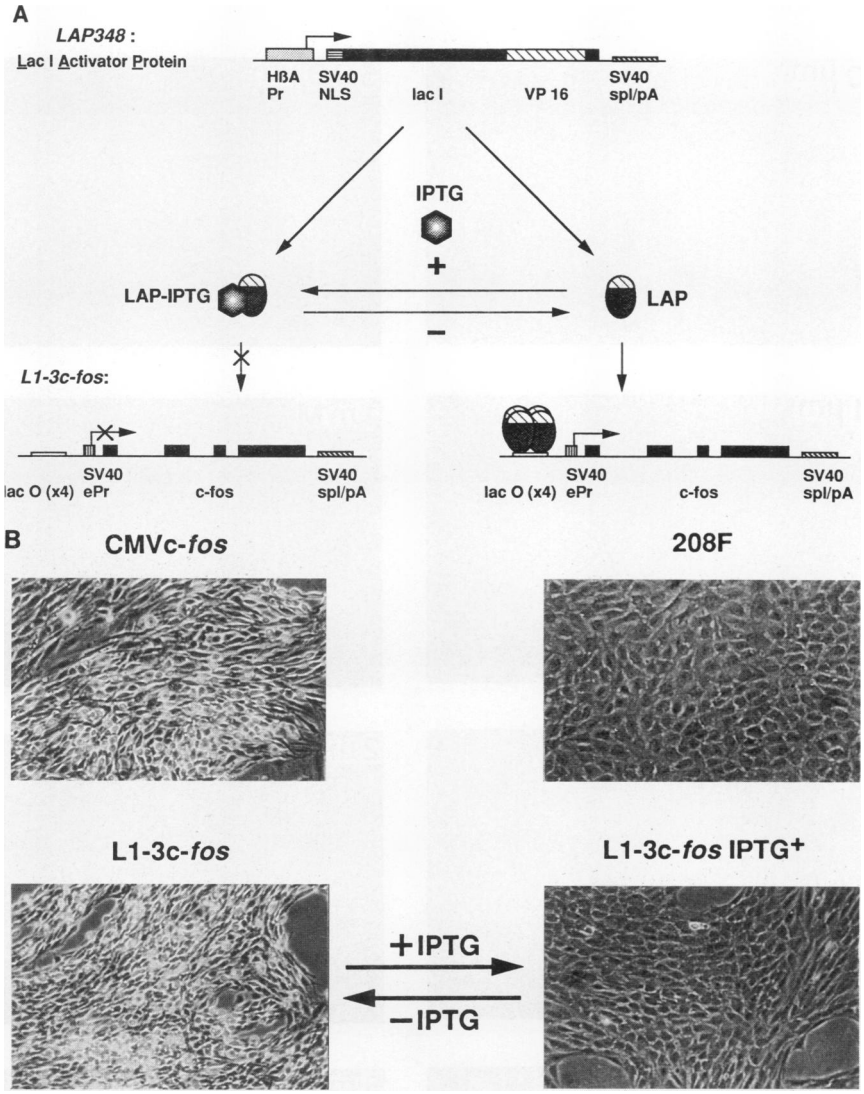
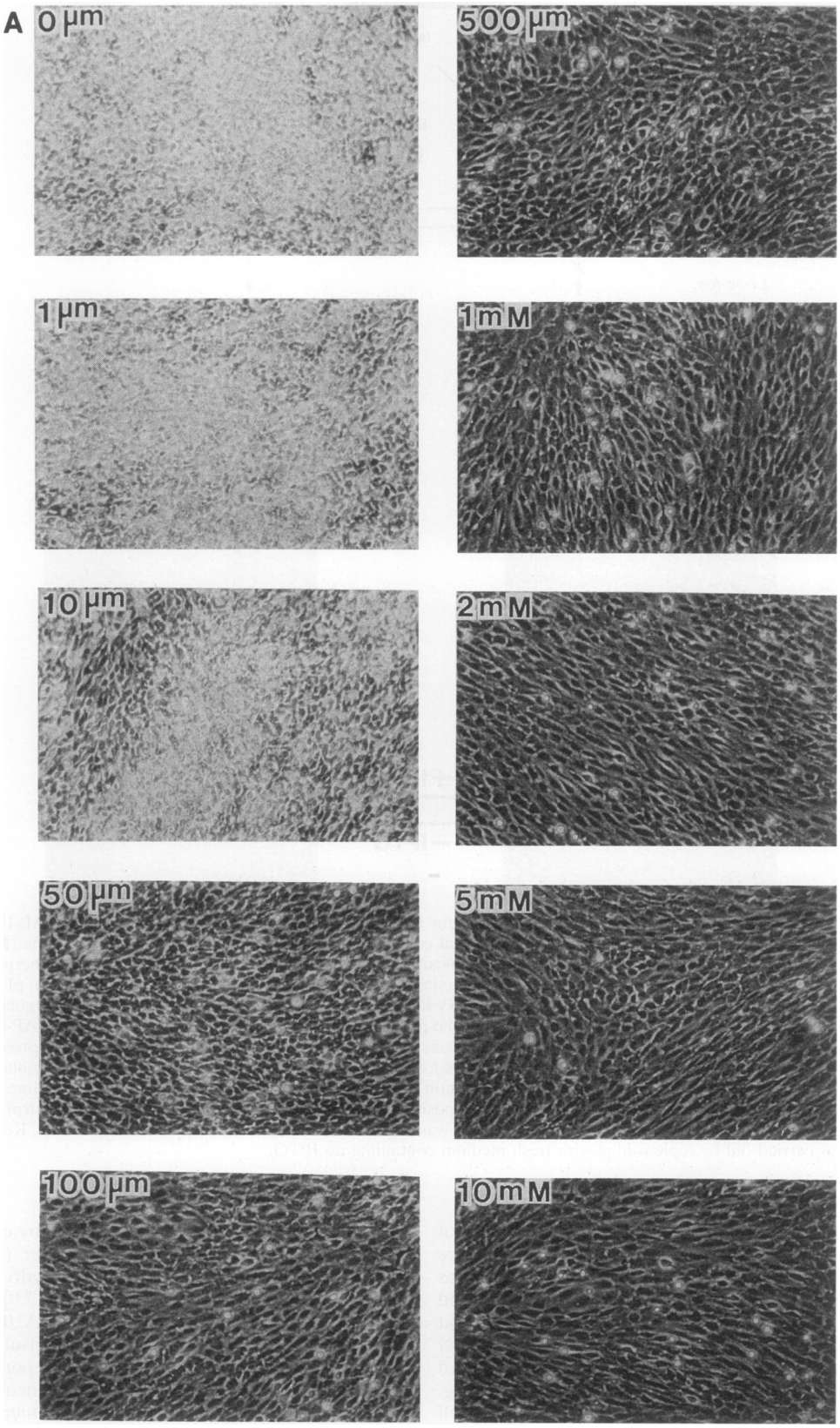


FIG. 1. Development of a conditional cell transformation system for c-Fos. (A) A schematic diagram of the LAP-IPTG reversible gene expression system adapted for c-*fos*. LAP (44) is a fusion protein that contains the *lac* repressor encoded by the *lacI* gene (■), the simian virus 40 (SV40) nuclear localization signal (NLS, ▤), and the potent eucaryotic transactivation domain VP16 (▧) from herpes simplex virus. The LAP gene in pLAP348 is expressed constitutively from a human β-actin promoter (HβAPr, ■). The c-*fos* (■) gene in pL1-3c-*fos* is controlled by four copies of the *lac* operator sequences (lac O, ▭) and by the SV40 early basal promoter (SV40 ePr, ▥). IPTG regulates the DNA binding activity of LAP and consequently controls the expression of c-*fos*. The presence of IPTG causes formation of inactive LAP-IPTG complexes and leads to the repression of *fos* expression. The absence of IPTG generates free LAP that binds to DNA and activates *fos* expression. (B) Conditional cell transformation by c-*fos*. pCMVc-*fos* alone or pLAP348, pL1-3c-*fos*, and pL17*neo* were cotransfected into rat fibroblast 208F cells. Clonal *fos*-transformed stable cell lines were obtained through G418 selection and cell subclonings. CMVc-*fos* is a stable cell line transformed by c-*fos* driven by the CMV promoter. This transformed cell line serves as a positive control for *fos* expression and a negative control for IPTG regulation. L1-3c-*fos* is a LAP-regulated *fos*-transformed stable cell line. IPTG was added directly to the culture medium at 2 mM. Removal of IPTG from the reverted cells was carried out by replenishing with fresh medium containing no IPTG.

dine (40 to 60 Ci/mmol; Amersham) at a final concentration of 10 μCi/ml for different lengths of time as indicated in the figure legends. Cells were washed twice with PBS, and the DNA was precipitated in situ, using 10% trichloroacetic acid. The fixed cells were rinsed twice with methanol and allowed to dry at room temperature. DNA was dissolved in 1 ml of 0.02 N NaOH, and the radioactivity (counts per minute) incorporated in each sample was quantitated by liquid scintillation counting.

Cell extracts and electrophoretic mobility shift assay. Cell extracts were prepared by the method of Thayer and Weintraub (81), with modification. Briefly, culture cells were washed twice with ice-cold PBS and harvested by centrifugation. Cell pellets were resuspended in lysis buffer (0.3 to 0.5 ml per 150-mm-diameter dish) (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 0.5 M NaCl, 1.5 mM Mg_2Cl, 20% glycerol, 0.2 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 2 μg of leupeptin per ml, 0.15 U of aprotinin per ml, 1 mM sodium fluoride, 1 mM sodium molybdate) and incubated at 4°C for 15 min. The cell lysates were cleared in a microcentrifuge at 4°C for 15 min, and the supernatants were dialyzed against lysis buffer containing 10 mM NaCl. Protein concentration was


A
0 μm
500 μm
1 μm
1 mM
10 μm
2 mM
50 μm
5 mM
100 μm
10 mM

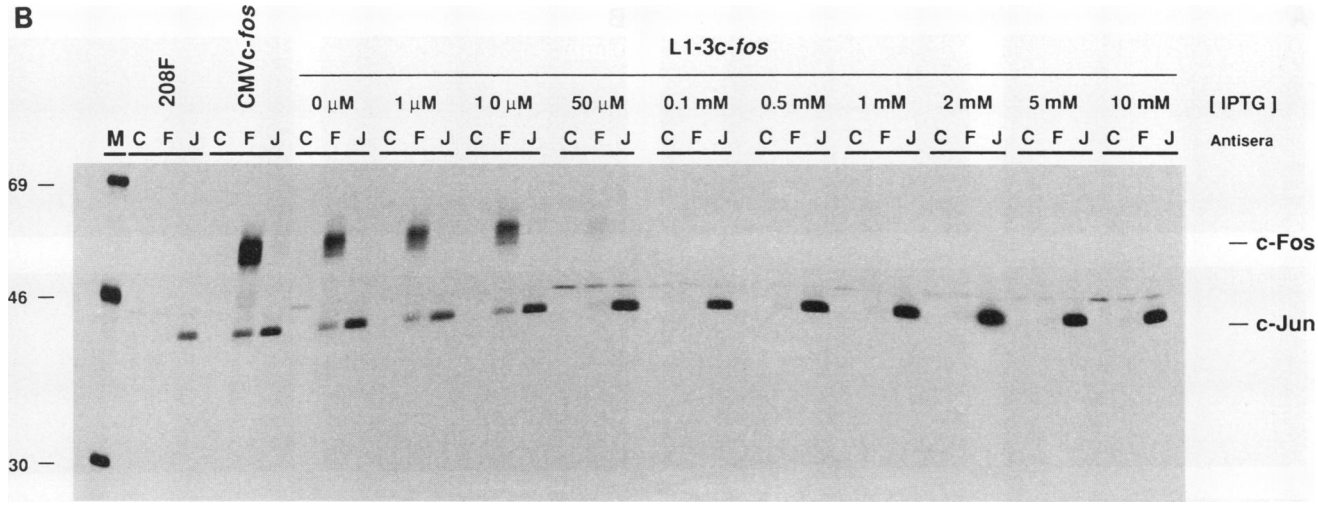


FIG. 2. Effect of IPTG concentration on reversion of cell transformation and repression of Fos expression. (A) L1-3c-*fos* transformed cells were exposed to 1 μM to 10 mM IPTG. The phase-contrast photomicrographs of cell morphology were taken 5 days following IPTG treatment. (B) IPTG was added to L1-3c-*fos* transformed cells at the indicated concentrations. [^{35}S]methionine-labeled cell extracts were prepared 5 days after IPTG addition and were treated with control antiserum (C), anti-c-Fos antiserum (F), and anti-c-Jun antiserum (J). Protein samples were analyzed on SDS–9% polyacrylamide gels. The positions of Fos and Jun proteins are indicated by arrows. Parental 208F cells and CMVc-*fos* transformed cells were used as controls for Fos expression. M denotes molecular weight markers, whose sizes (in kilodaltons) are indicated at the left.

determined by the method of Bradford (Bio-Rad protein assay). Extracts of different cell samples were adjusted to the same protein concentration with the dialysis buffer and stored in aliquots in liquid nitrogen.

Electrophoretic mobility shift assays were performed essentially as described previously (90). Cell extracts (16 μg) were preincubated in binding buffer (10 mM HEPES [pH 7.9], 50 mM NaCl, 5 mM Mg_2Cl, 5% glycerol, 5% sucrose, 1 mM EDTA, 5 mM dithiothreitol) at 37°C for 10 min in a final volume of 10 μl, followed by the addition of 1 μg poly(dI-dC) · poly(dI-dC) for 10 min at room temperature. α-^{32}P-labeled AP-1 oligonucleotide probe (66) (0.4 ng) was then added, and the samples were incubated for an additional 10 min at room temperature. In supershift assays, 1 μl of Fos antibody (anti-wbFos) (1) was added to the reaction, following the addition of the AP-1 probe. DNA-protein complexes were resolved on 4.5% native Tris-glycine polyacrylamide gels, dried, and visualized by autoradiography.

Isolation of RNA and Northern (RNA) analysis. Total RNA was isolated by the method of acid-guanidinium-phenol-chloroform (9), with modification. Briefly, culture cells in 150-mm-diameter dishes were lysed in situ with 3 ml of lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl, 0.1 M β-mercaptoethanol). The lysates were extracted once with 1 volume of water-saturated phenol, 0.2 volume of chloroform (chloroform-isoamylalcohol, 49:1), and 0.1 volume of 2 M sodium acetate (pH 4.0). Phenol-chloroform extractions were carried out three more times in the absence of sodium acetate. RNA was precipitated with 1 volume of isopropanol, and the samples were incubated at −20°C overnight. The RNA pellet was resuspended in 0.5 ml of lysis buffer and 0.5 ml of isopropanol, reprecipitated at −20°C for longer than 1 h, and dissolved in H_2O.

RNA analysis was performed by Northern hybridization. RNA samples (10 μg) were prepared in 1× morpholinepropanesulfonic acid (MOPS)–2.2 M formaldehyde–50% formamide–0.05 μg of ethidium bromide per μl, denatured at 65°C for 10 min, and electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde and 1× MOPS buffer. RNA was transferred onto a nylon membrane (Hybond-N; Amersham) in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and immobilized to the membrane with a UV cross-linker (Stratagene). Blots were prehybridized at 37°C for 2 h and hybridized to [α-^{32}P]dCTP-labeled cDNA probes (Oligolabelling kit; Pharmacia) in a hybridization solution containing 50% formamide, 5× Denhardt's solution, 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 8.0]), 1% SDS, and 100 μg of denatured salmon sperm DNA per ml at 37°C for 24 h. The blots were then washed twice in 2× SSC–0.1% SDS at room temperature for 15 min, washed once in 0.1× SSC–0.1% SDS at 55°C for 60 min, and visualized by autoradiography. Radioactive probes were stripped from the membranes by submerging the membranes in a solution of boiling 0.1% SDS and cooling to room temperature.

RESULTS

Conditional regulation of c-*fos* expression. Conditional regulation of gene activity can be achieved at either the posttranslational or transcriptional level. For example, fusion to the hormone-binding domain of the glucocorticoid or estrogen receptors confers hormone-sensitive function to the gene product (64). Alternatively, a gene of interest can be linked to an inducible promoter, allowing expression to be regulated by an inducing agent. To achieve conditional expression of c-*fos*, we used a conditional gene expression strategy based on the LAP and IPTG (44). This strategy was chosen because it offers several advantages over other inducible systems (44, 88). Most notably, gene expression is controlled effectively by an inert sugar analog, IPTG, which has no apparent effect on cellular metabolism at the concentrations used. As illustrated in Fig. 1A, expression of *fos* is activated by binding of LAP to *lac* operator sequences placed upstream of the c-*fos* gene. The presence of IPTG results in the formation of inactive LAP-IPTG complexes that are unable to bind DNA and activate *fos* expression. A distinct feature of this system is that it functions by inducible repression of transcription. Therefore, in contrast

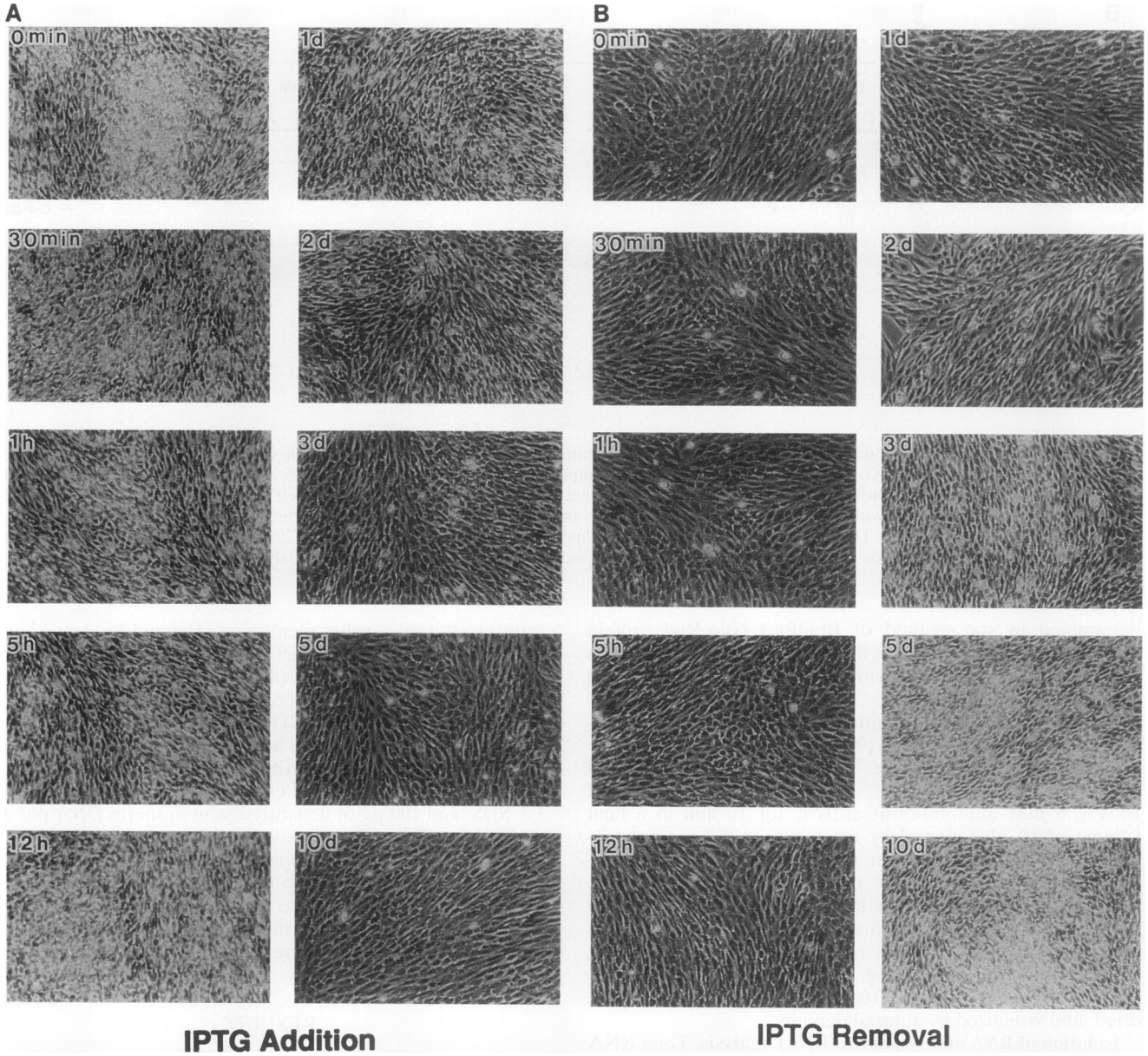


FIG. 3. Time course of IPTG-induced morphological reversion and transformation in L1-3c-*fos* cells. (A) L1-3c-*fos* transformed cells were cultured in the presence of IPTG for different lengths of time from 30 min to 10 days (d). Photomicrographs of cell morphology were taken after each incubation time as indicated. (B) L1-3c-*fos* reverted cells were deprived of IPTG through medium changes for different periods of time as indicated. Photomicrographs of cell morphology corresponding to each time length are shown.

to many other inducible systems, there is little breakthrough expression in the repressed state.

To establish stable cell lines that express Fos under the control of LAP-IPTG, the effector plasmid pLAP348, which constitutively expresses LAP from a human β-actin promoter (44), was cotransfected with pL1-3c-*fos* and pL17*neo* into 208F rat fibroblasts in the absence of IPTG. This cell line has been most frequently used in the studies of cell transformation by *fos* (12). Under normal growth conditions, 208F cells do not express detectable levels of the endogenous c-Fos protein. Transformed cells were identified on the basis of their distinctive morphology. In general, these cells were spherical or spindle shaped, refractile in appearance, and disorganized in arrangement. Furthermore, they tended to form multiple cell layers, whereas normal 208F cells grew as a flat contact-inhibited monolayer. The cell lines isolated initially were heterogeneous, consisting of both normal and transformed cells. Following several rounds of subcloning, clonal transformed cell lines were obtained (L1-3c-*fos* cells). Clonal cell lines transformed by c-*fos* under the control of a CMV promoter (CMVc-*fos* cells) were also generated to serve as a negative control for LAP-IPTG regulation and as a positive control for *fos* expression.

As shown in Fig. 1B, the parental 208F cells grew as a flat nonrefractile monolayer, whereas L1-3c-*fos* and CMVc-*fos* cells displayed a transformed morphology. To examine

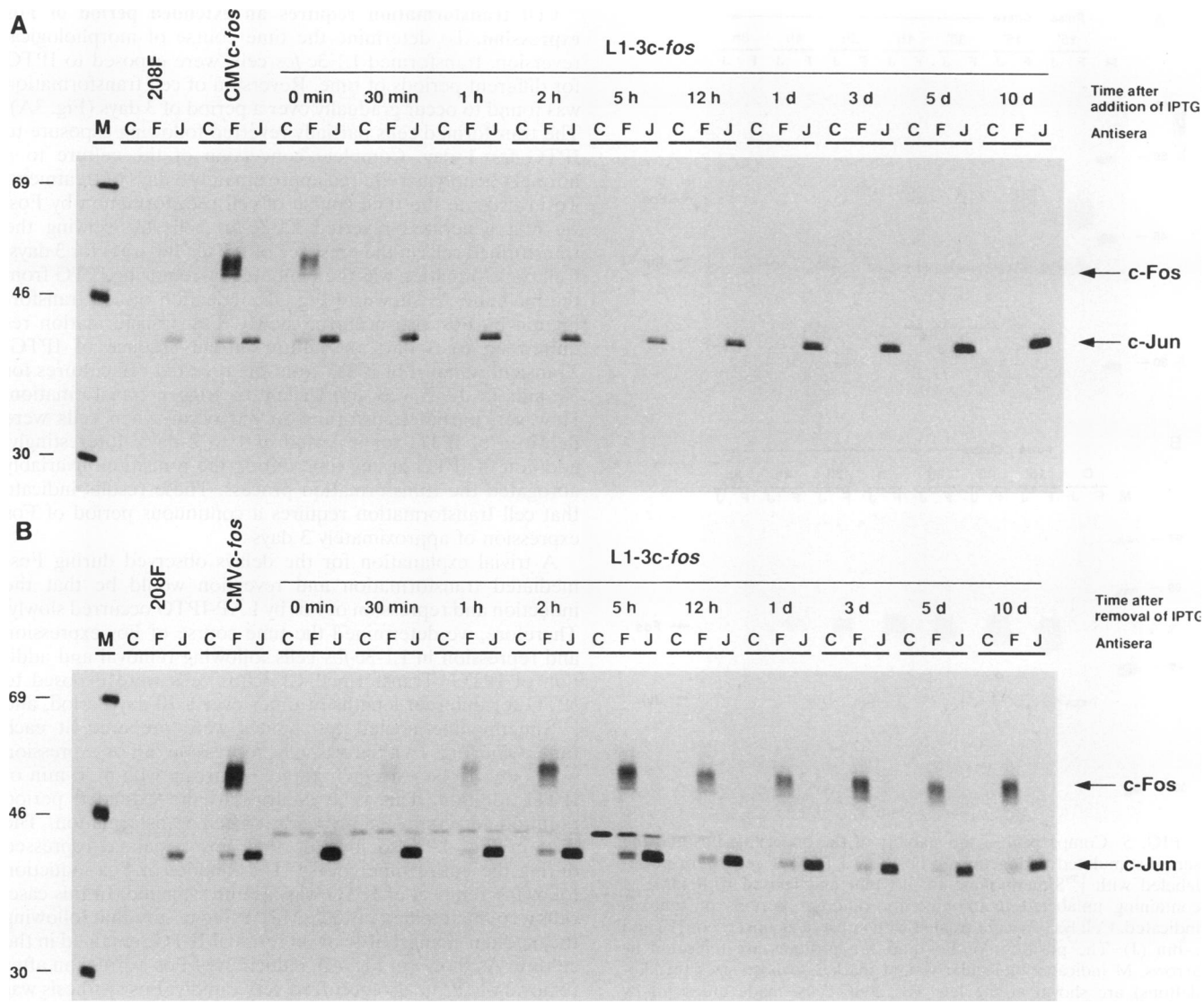


FIG. 4. Kinetics of Fos repression and expression in response to IPTG. (A) Time course of Fos repression after addition of IPTG. L1-3c-*fos* cells were exposed to IPTG for different periods of time as indicated. ^{35}S-labeled cell extracts were prepared at different time points and treated with control serum (C), anti-c-Fos antiserum (F), and anti-c-Jun antiserum, and the products were analyzed on SDS–9% polyacrylamide gels. The positions of Fos and Jun proteins are indicated by arrows. Parental 208F cells and CMVc-*fos* transformed cells were used as controls for Fos expression. Sizes (in kilodaltons) of molecular weight markers are indicated at the left. (B) Time course of Fos expression after removal of IPTG. L1-3c-*fos* transformed cells were reverted in the presence of IPTG (100 μM) for 3 days. IPTG was then removed from reverted cultures for different periods of time. The cells were harvested and analyzed as described above.

whether transformed L1-3c-*fos* cells could be reverted to a normal phenotype, we added IPTG to the culture medium. Addition of IPTG to either 208F cells or CMVc-*fos* transformed cells had no apparent effect on cell morphology. This finding is consistent with previous findings demonstrating that low concentrations of IPTG have no toxic effect (44, 88) and with the fact that the CMV promoter is not regulated by IPTG. In contrast, the addition of IPTG to transformed L1-3c-*fos* cells resulted in reversion to a normal phenotype characteristic of 208F cells. The reverted state was stable through multiple cell divisions, and the cultures could be maintained as flat monolayers as long as IPTG was present in the medium. However, removal of IPTG from the culture resulted in cell transformation. All of the three independently derived L1-3c-*fos* cell lines tested displayed this property, whereas none of the CMVc-*fos* transformed cell lines responded to IPTG.

To determine the effective IPTG concentration, transformed L1-3c-*fos* cells were cultured in the presence of 1 μM to 10 mM IPTG (Fig. 2A). Morphological reversion was monitored for up to 5 days after administration of IPTG. As little as 100 μM IPTG was found to completely reverse *fos*-induced transformation. This is substantially lower than the concentrations previously reported for repression of other IPTG-regulated genes (44, 92). Concentrations of up to 10 mM IPTG were equally effective, with no apparent cytotoxicity. Therefore, 2 mM IPTG was selected for the subsequent studies unless otherwise indicated. It should be noted that lower concentrations of IPTG, e.g., 50 μM, appeared to partially revert the

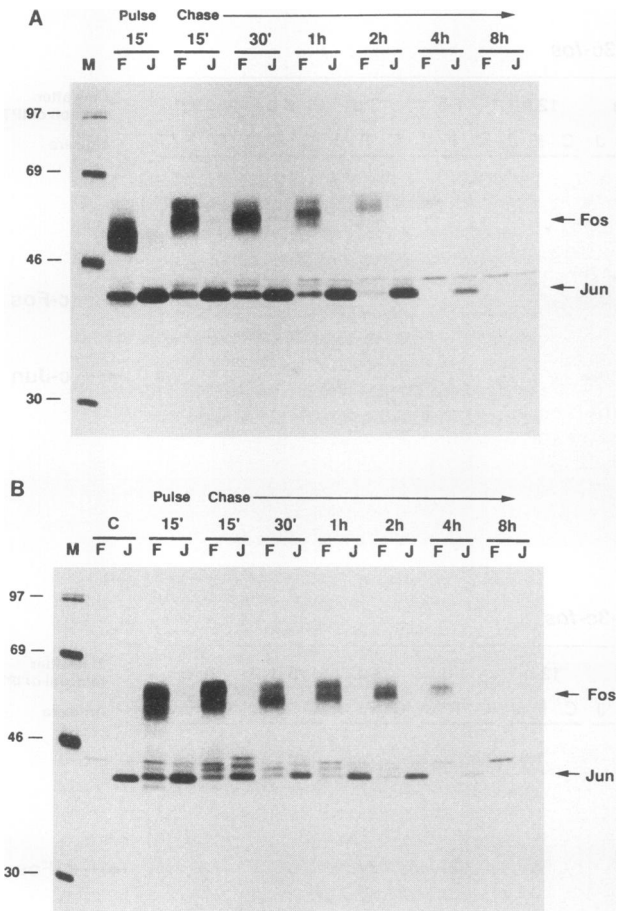


FIG. 5. Comparison of the stability of the oncogenic Fos and the serum-stimulated endogenous c-Fos. (A) L1-3c-*fos* cells were pulse-labeled with [^{35}S]methionine for 15 min and chased with DMEM containing unlabeled methionine for different periods of time as indicated. Cell lysates were treated with antisera against c-Fos (F) and c-Jun (J). The positions of Fos and Jun proteins are indicated by arrows. M indicates molecular weight markers, whose sizes (in kilodaltons) are shown at the left. (B) 208F cells, made quiescent by incubation in 0.5% FBS for 48 h, were stimulated with 20% dialyzed FBS for 30 min. Pulse-chase immunoprecipitation analysis was conducted as described in the text. C, a cell lysate from 208F cells maintained under normal growth conditions.

transformed cell morphology. Therefore, morphological conversion of L1-3c-*fos* cells was dependent on the concentration of IPTG in the medium and occurred across a wide range of IPTG concentrations.

The level of Fos expressed in L1-3c-*fos* cells cultured in the presence of various concentrations of IPTG was found to correspond well with the cell phenotype (Fig. 2B). Pulse-labeling immunoprecipitation experiments revealed that 1 or 10 μM IPTG had little effect on Fos synthesis, 50 μM IPTG significantly reduced expression, and concentrations of 100 μM and above completely inhibited Fos expression (Fig. 2B). Thus, the apparent degree of morphological transformation was dictated by the amount of Fos expressed. There appeared to be a critical threshold level of Fos that was required for cell transformation. Figure 2B also illustrates that there is a relatively high level of c-Jun present in normal 208F cells. This may explain, in part, the susceptibility of 208F cells to transformation by Fos.

Cell transformation requires an extended period of Fos expression. To determine the time course of morphological reversion, transformed L1-3c-*fos* cells were exposed to IPTG for different periods of time. Reversion of cell transformation was found to occur gradually over a period of 3 days (Fig. 3A). The transformed cells partially reverted following exposure to IPTG for 1 day. Complete conversion of the culture to a normal phenotype required approximately 3 days of treatment. To investigate the time course of cell transformation by Fos, we first generated reverted L1-3c-*fos* cells by growing the transformed cells in the presence of IPTG (100 μM) for 3 days. Cell transformation was then initiated by removing IPTG from the medium. As shown in Fig. 3B, induction of cell transformation by Fos also occurred slowly. Full transformation required up to 3 days of culture in the absence of IPTG. Transient removal of IPTG from the reverted cell cultures for 30 min to 12 h was insufficient to trigger transformation. However, partial transformation did occur when cells were deprived of IPTG for a period of 1 to 2 days. Interestingly, addition of IPTG at any time during the transition invariably abrogated the transformation process. These results indicate that cell transformation requires a continuous period of Fos expression of approximately 3 days.

A trivial explanation for the delays observed during Fos-mediated transformation and reversion would be that the induction and repression of Fos by LAP-IPTG occurred slowly. Therefore, we determined the time course of Fos expression and repression in L1-3c-*fos* cells following removal and addition of IPTG. Transformed L1-3c-*fos* cells were exposed to IPTG for different lengths of times over a 10-day period, and [^{35}S]methionine-labeled cell lysates were prepared at each time point (Fig. 4A). Surprisingly, repression of Fos expression was found to be extremely rapid, occurring within 30 min of IPTG addition. This is in contrast to the extended period required for complete reversion of cell transformation. The data in Fig. 4A also indicate that Fos remained repressed during the entire time course. The kinetics of Fos induction following removal of IPTG was also investigated. In this case, cells were maintained in 100 μM IPTG to ensure that following the medium change, little or no residual IPTG remained in the culture. As shown in Fig. 4B, induction of Fos expression after removal of IPTG also occurred very rapidly. Fos synthesis was detectable within 30 min and reached peak levels 2 h after IPTG removal. These data thus confirm the observation that cell transformation requires an extended period of Fos expression.

The preceding experiments relate the rate of Fos synthesis to the presence of IPTG. It is at least conceivable that IPTG also modulates the turnover rate of Fos and that this could affect its ability to induce transformation. Previously, it was suggested that mutations that occurred during the generation of the v-*fos* gene stabilize its protein product and thereby enhance its transforming potential (62). Therefore, we compared the half-life of Fos in L1-3c-*fos* cells with that of c-Fos in serum-stimulated fibroblasts in pulse-chase immunoprecipitation experiments (Fig. 5). To obtain an accurate measurement of the half-life of Fos in L1-3c-*fos* cells, IPTG was added to the culture medium during the chase period to prevent further Fos synthesis. Cell lysates, prepared at each of the indicated time points, were treated with anti-Fos and anti-Jun antisera, and the products were analyzed on SDS-gels (Fig. 5A). Newly synthesized Fos appeared as an broad band of approximately 55 kDa because of heterogeneous phosphorylation (13). It was rapidly converted to more highly modified forms that migrated with an apparent molecular mass of 60 kDa. The amount of radioactivity in each Fos band was measured and plotted

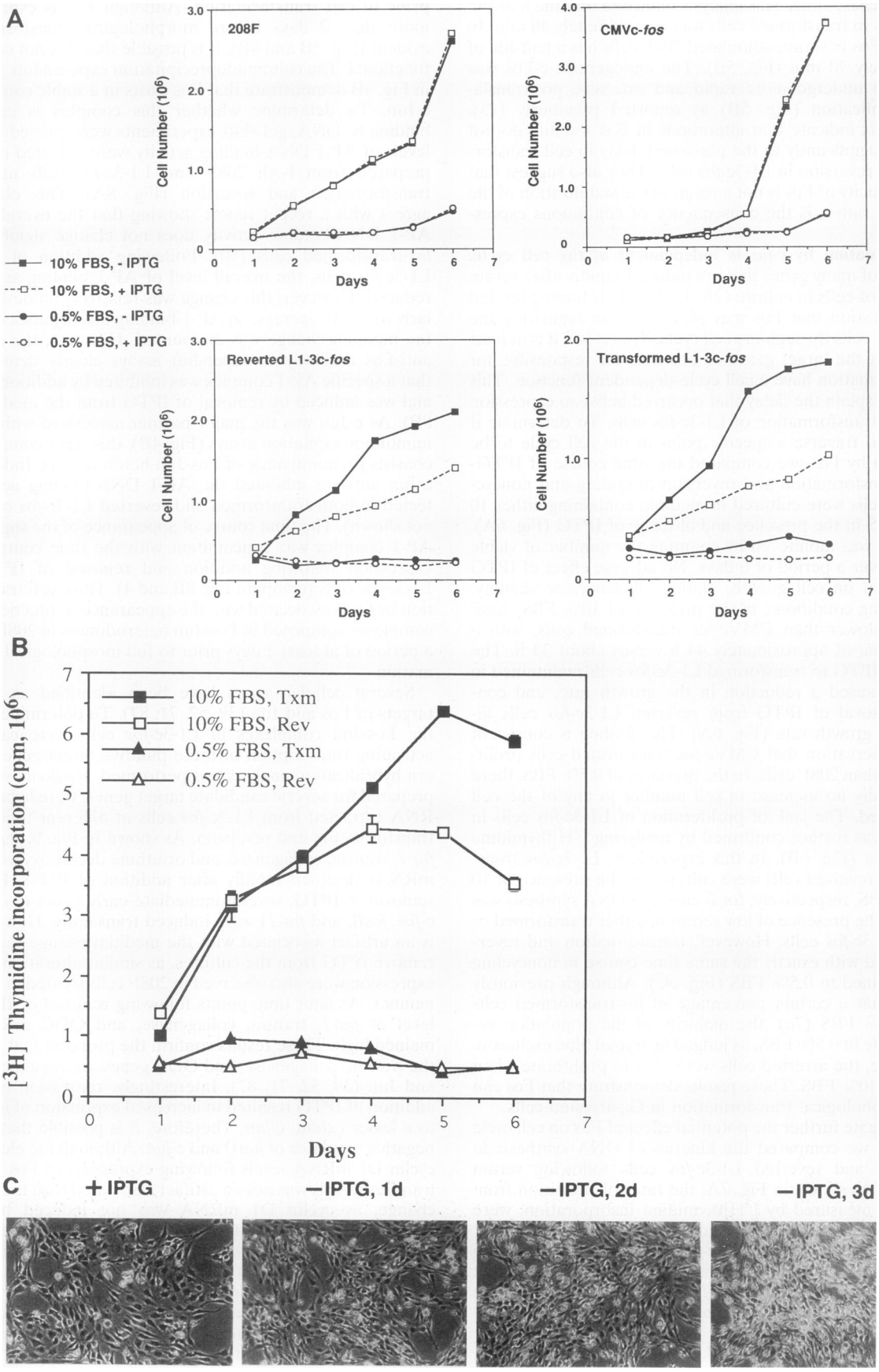
against the chase time. This analysis indicated that the half-life of the c-Fos in transformed cells was approximately 40 min. In contrast, c-Fos in serum-stimulated 208F cells has a half-life of approximately 70 min (Fig. 5B). The endogenous c-Fos was observed to undergo more rapid and extensive posttranslational modification (Fig. 5B) as reported previously (13). These results indicate that alterations in Fos stability do not contribute significantly to the perceived delay in cell transformation and reversion in L1-3c-*fos* cells. They also suggest that the oncogenicity of Fos is not a result of the stabilization of the protein but rather is the consequence of continuous expression.

Transformation by c-*fos* is independent of the cell cycle. c-*fos* is one of many genes that are induced rapidly after serum stimulation of cells in culture (28, 43, 57). This finding has led to the suggestion that Fos may play a role in regulating the transition of cells through the cell cycle. Therefore, it is at least possible that the target genes of Fos that are responsible for cell transformation have a cell cycle-dependent function. This might also explain the delay that occurred between expression of Fos and transformation of L1-3c-*fos* cells. To determine if cells need to traverse a specific point in the cell cycle to be transformed by Fos, we compared the time course of IPTG-induced transformation and reversion in cycling and noncycling cells. Cells were cultured in medium containing either 10 or 0.5% FBS in the presence and absence of IPTG (Fig. 6A). Cell growth was monitored by counting the number of viable cells daily over a period of 6 days. No adverse effect of IPTG was observed on cell growth, plating efficiency, or viability. Under cycling conditions, in the presence of 10% FBS, 208F cells grew slower than CMVc-*fos* transformed cells, with a doubling time of approximately 44 h versus about 33 h. The addition of IPTG to transformed L1-3c-*fos* cells maintained in 10% FBS caused a reduction in the growth rate, and conversely, removal of IPTG from reverted L1-3c-*fos* cells increased the growth rate (Fig. 6A). This finding is consistent with the observation that CMVc-*fos* transformed cells proliferate faster than 208F cells. In the presence of 0.5% FBS, there was essentially no increase in cell number in any of the cell lines analyzed. The lack of proliferation of L1-3c-*fos* cells in 0.5% FBS was further confirmed by analyzing [^{3}H]thymidine incorporation (Fig. 6B). In this experiment, L1-3c-*fos* transformed and reverted cells were cultured in the presence of 10 and 0.5% FBS, respectively, for 6 days. No DNA synthesis was detected in the presence of low serum in either transformed or reverted L1-3c-*fos* cells. However, transformation and reversion occurred with exactly the same time course in noncycling cells maintained in 0.5% FBS (Fig. 6C). Although previously we found that a certain percentage of *fos*-transformed cells died in 0.1% FBS (76), the majority of the population remained viable in 0.5% FBS, as judged by trypan blue exclusion. Furthermore, the arrested cells were able to proliferate when returned to 10% FBS. These results demonstrate that Fos can induce morphological transformation in G_0-arrested cells.

To investigate further the potential effect of Fos on cell cycle progression, we compared the kinetics of DNA synthesis in transformed and reverted L1-3c-*fos* cells following serum stimulation. As shown in Fig. 7A, the rates of transition from G_0 to S, as measured by [^{3}H]thymidine incorporation, were similar in transformed or reverted L1-3c-*fos* cells and in 208F cells. In addition, induction of c-*fos* expression, by removing IPTG from the culture medium, failed to drive quiescent L1-3c-*fos* cells into S phase (Fig. 7B). Taken together, these data indicate that Fos expression by itself is not sufficient to stimulate cell cycle progression.

Fos binds to DNA and regulates target gene expression prior to cell transformation. Although Fos is expressed for more than 2 days before morphological transformation is evident (Fig. 3B and 4B), it is possible that it is not completely functional. The coimmunoprecipitation experiments presented in Fig. 4B demonstrate that Fos exists in a stable complex with c-Jun. To determine whether this complex is capable of binding to DNA, gel shift experiments were carried out. High levels of AP-1 DNA-binding activity were detected in extracts prepared from both 208F and L1-3c-*fos* cells undergoing transformation and reversion (Fig. 8A). This observation agrees with a recent report showing that the overall level of AP-1 DNA-binding activity does not change significantly in *fos*-transformed cells (30). Following addition of IPTG to L1-3c-*fos* cells, the overall level of AP-1 binding activity was reduced. However, this change was relatively modest, particularly when the increase in AP-1-binding activity associated with the medium change was discounted. However, the use of an anti-Fos antibody in supershift assays clearly demonstrated that a specific AP-1 complex was inhibited by addition of IPTG and was induced by removal of IPTG from the medium (Fig. 8B). As c-Jun was the major partner associated with c-Fos in immunoprecipitation assays (Fig. 4B), this AP-1 complex likely consists predominantly of Fos-Jun heterodimers. Indeed, anti-c-Jun antisera inhibited the AP-1 DNA-binding activity detected in both transformed and reverted L1-3c-*fos* cells (data not shown). The time course of appearance of the supershifted AP-1 complex was concomitant with the time course of Fos expression following addition and removal of IPTG from L1-3c-*fos* cells (compare Fig. 8B and 4). Thus, cell transformation by *fos* is associated with the appearance of functional AP-1 complexes composed of Fos-Jun heterodimers in 208F cells for a period of at least 2 days prior to full morphological transformation.

Several cellular genes have been identified as potential targets of Fos and Jun (39, 52, 71, 87). To determine whether the Fos-Jun complexes in L1-3c-*fos* cells were capable of activating transcription of these putative target genes, Northern hybridization analysis was performed. Radioactive probes, prepared for several candidate target genes, were hybridized to RNA extracted from L1-3c-*fos* cells at different times during transformation and reversion. As shown in Fig. 9, the level of *fra-1*, transin, collagenase, and ornithine decarboxylase (ODC) mRNAs declined rapidly after addition of IPTG. Following removal of IPTG, several immediate-early genes (c-*jun*, *junB*, c-*fos*, *fosB*, and *fra-1*) were induced transiently. However, this is an artifact associated with the medium change required to remove IPTG from the cultures, as similar alterations in gene expression were also observed in 208F cells treated in the same manner. At later time points following removal of IPTG, the level of *fra-1*, transin, collagenase, and ODC mRNAs remained high. These results confirm the proposal that *fra-1* and the transin, collagenase, and ODC genes are regulated by Fos and Jun (39, 52, 71, 87). Interestingly, repression of Fos by addition of IPTG resulted in increased expression of *junD* and, to a lesser extent, c-*jun*. Therefore, it is possible that Fos is a negative regulator of *junD* and c-*jun*. Although the elevation in cyclin D1 mRNA levels following expression of Fos occurred transiently, this was not an artifact associated with the medium change, as cyclin D1 mRNA was not induced by serum stimulation. In contrast, cyclin D3 mRNA was expressed constitutively at high levels in both reverted and transformed L1-3c-*fos* cells. Taken together, these data demonstrate that Fos expression rapidly induces the synthesis of *fra-1*, transin, collagenase, and ODC mRNAs and repression of c-*fos* results in a rapid decrease in the synthesis of these mRNAs. Therefore, transcriptionally active Fos-Jun complexes are present for


A
208F
CMVc-fos
Reverted L1-3c-fos
Transformed L1-3c-fos
Cell Number (10^6)
Days
10% FBS, - IPTG
10% FBS, + IPTG
0.5% FBS, - IPTG
0.5% FBS, + IPTG
B
[3H] Thymidine incorporation (cpm, 10^6)
10% FBS, Txm
10% FBS, Rev
0.5% FBS, Txm
0.5% FBS, Rev
Days
C
+ IPTG
−IPTG, 1d
−IPTG, 2d
−IPTG, 3d

at least 2 days before L1-3c-*fos* cells become fully transformed. Furthermore, activation of the Fos target genes, *fra-1* and the transin, collagenase, and ODC genes, is not sufficient to trigger morphological transformation immediately.

DISCUSSION

One of the unique features of the *fos* oncogene is that it is exclusively associated with the induction of bone and cartilage tumors in mice. Both the FBJ and FBR viruses induce osteogenic sarcoma, and transgenic mice expressing an exogenous c-*fos* gene develop osteosarcomas or chondrosarcomas although *fos* is often expressed at similar levels in many other tissues (21, 22, 29, 83). Interestingly, the endogenous c-*fos* gene is expressed in both osteoblasts and chondrocytes (29, 75). However, it is not expressed in the precursor cells of the periosteum from which virus-induced tumors are believed to arise (12, 75). A null mutation of the endogenous c-*fos* gene introduced by homologous recombination in embryonal stem cells results in a severe defect in bone development in mice (38, 84). The mutant mice develop osteopetrosis with deficiencies in bone remodeling and tooth eruption. Therefore, oncogenesis may be a consequence of the inappropriate expression of a transcription factor, Fos, that is required for later stages of bone development in a precursor cell that retains some capacity for osteoid and chondroid differentiation. This proposal implies that there is a specific target(s) for Fos in these cells that mediates its transforming activity.

Although the FBR v-*fos* gene is capable of inducing morphological transformation of bone and cartilage cells in culture, the transformed cells are difficult to maintain in long-term culture (36). Like many retroviral oncogenes, *fos* has been shown to induce transformation of cultured fibroblast cell lines. This provides a convenient assay for biological activity and represents an in vitro analog of oncogenesis. Although it is not proven that Fos affects the same target gene(s) in vitro and in vivo, this model system has been of great utility in the study of several other oncogenes, and it is reasonable to assume that there is a meaningful relationship between morphological transformation in vitro and tumor formation in vivo. In the case of the *fos* oncogene, a rat fibroblast cell line termed 208F (65), which was derived from Rat-1 cells, has been shown to be particularly susceptible to transformation by v-*fos* (16). It was demonstrated that in this cell line, continuous expression of the normal c-*fos* gene results in transformation (54). Although the various mutations that have occurred in the FBJ and FBR v-*fos* genes enhance transforming activity, none are absolutely essential. Therefore, we developed a conditional expression system for c-*fos* to investigate its transforming activity in 208F cells.

Conditional transformation by Fos. Conditional oncogene alleles have provided a valuable tool for investigation of the molecular basis of oncogenesis (67). Previously, Fos-estrogen receptor fusion proteins, in which Fos function was dependent on the presence of a steroid hormone, were used to investigate cell transformation (72, 79). However, there are two problems associated with this approach. First, fusion of the hormone-binding domain of the estrogen receptor alters the transcriptional properties of Fos (72). For example, fusion of ΔFosB to the estrogen receptor confers transforming activity to ΔFosB, whereas the native protein lacks this ability (58, 72). Second, steroid hormones may affect cell physiology and complicate analysis of the transformed cell phenotype.

To circumvent these shortcomings, we used an approach based on the use of an inert sugar analog, IPTG, to repress expression of an exogenous c-*fos* gene under the control of the LacI promoter. This approach was previously used successfully in the analysis of p53 (92). Induction and repression of c-Fos in this system in L1-3c-*fos* cells occurred very rapidly, within 30 min following removal or addition of IPTG to the culture medium. This rapid mode of *fos* regulation allowed precise determination of the time required for Fos to induce morphological transformation. Here we show that there is a time delay between the induction of Fos expression and full transformation of L1-3c-*fos* cells. This finding implies that the target(s) of Fos require an extended period to achieve a critical threshold level that is required for morphological conversion.

Fos target genes. Although transformation by Fos is a slow process, an additional AP-1 DNA-binding complex, composed predominantly of c-Fos and c-Jun, was formed rapidly immediately after Fos was expressed. This was accompanied by dramatic changes in the expression of several putative Fos target genes (Fig. 9) (39, 52, 71, 87). For example, the steady-state levels of mRNAs encoding Fra-1, transin, collagenase, and ODC were low or undetectable in normal cells but were induced within 30 min in cells expressing c-Fos. Repression of c-Fos expression led to a rapid and significant reduction in the expression of these genes. Fra-1 and transin mRNAs are also expressed highly in v-*fos*-transformed cells (72, 73, 86). Therefore, at least two of the known targets are shared by v-*fos* and c-*fos*. In addition to activating expression of several cellular genes, Fos also had a negative effect on the expression of *junD*, *fra-2*, and, to a lesser extent, c-*jun* (Fig. 9; data not shown). These results indicate that cell transformation by Fos was associated with both activation and repression of cellular genes. Furthermore, the finding that *fra-1* and *junD* are rapidly activated and repressed in response to Fos expression provides evidence for cross-regulation among AP-1 transcription factors. This implies that the ability of Fos to transform cells may depend on target genes that are regulated either directly or indirectly by Fos. Thus, Fos transformation could be a consequence of altered expression of a complex network of transcription factors.

Elevated expression of transin and ODC mRNAs also occurs in cells transformed by other oncogenes such as *ras*, *src*, the polyomavirus middle-T-antigen gene, and *fosB* (33, 34, 50, 51, 58). The activity of ODC has been recently shown to be required for *src*-mediated cell transformation (33). It has been proposed that extracellular matrix-degrading proteinases such as transin and collagenase play important roles in tumorigen-

FIG. 6. Analysis of growth properties of *fos*-transformed and nontransformed cells in high and low-serum conditions. (A) Cell growth rates for 208F, CMVc-*fos*, and reverted and transformed L1-3c-*fos* cells in high (10% FBS)- and low (0.5% FBS)-serum conditions in the presence and absence of IPTG. Cells were seeded at a density of 2×10^5 cells per 35-mm-diameter well in six-well plates. The number of viable cells were counted daily in duplicates for 6 days. Cell medium was renewed every 2 days. (B) Analysis of DNA synthesis in transformed and reverted L1-3c-*fos* cells cultured in high- and low-serum conditions. Transformed (Txm) and reverted (Rev) L1-3c-*fos* cells were seeded in six-well plates (2×10^5 cells per well). DNA synthesis at different time points of incubation was measured by labeling cells with [^{3}H]thymidine (10 μCi/ml) for 24 h. The results represent means of duplicate samples ± a standard deviation. (C) Transformation of G_0-arrested L1-3c-*fos* cells. Reverted L1-3c-*fos* cells were arrested in G_0 by incubation in 0.5% FBS for 48 h. IPTG was then removed from the culture for different periods of time (days [d]), as indicated at the top. The cell cultures were maintained in 0.5% FBS for the entire experiment.

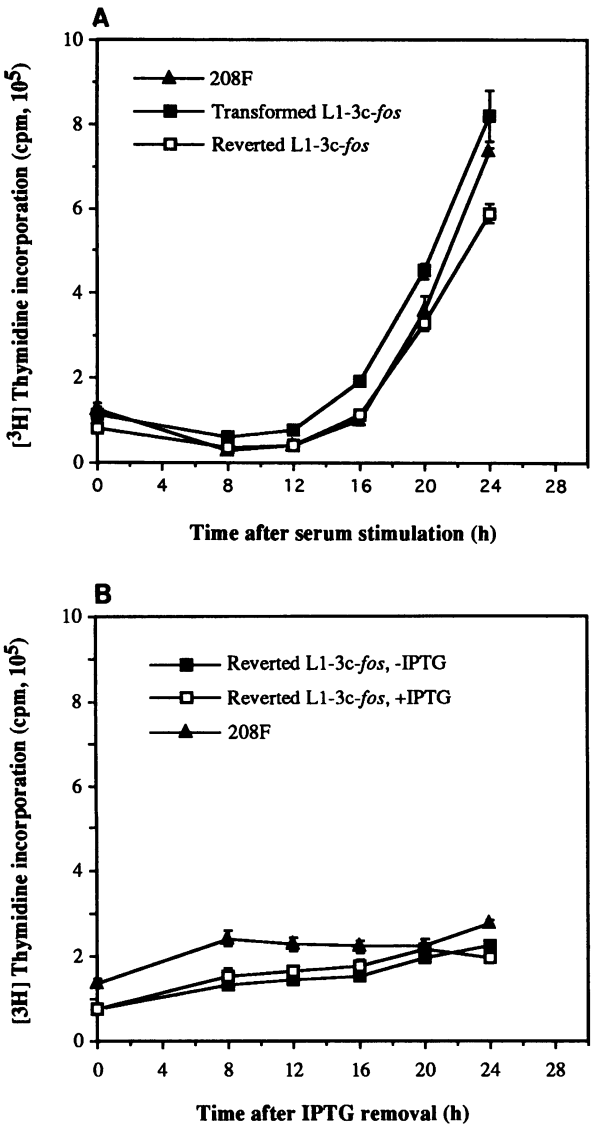


FIG. 7. Effect of Fos expression on cellular DNA synthesis. (A) [^{3}H]thymidine incorporation following serum stimulation of quiescent transformed and reverted L1-3c-*fos* and 208F cells. Cells, rendered quiescent by culturing in 0.5% FBS for 48 h, were stimulated with 20% FBS for different times as indicated. The cultures were labeled with [^{3}H]thymidine (10 μCi/ml) during the last 8 h of serum stimulation. (B) [^{3}H]thymidine incorporation following removal of IPTG from quiescent reverted L1-3c-*fos* cells. Quiescent reverted L1-3c-*fos* cells cultured in 0.5% FBS were deprived of IPTG (−IPTG) or were subject to medium change containing IPTG (+IPTG) for different lengths of time. Quiescent 208F cells were used as a control to monitor the effect of medium change (0.5% FBS) on [^{3}H]thymidine incorporation. Cell cultures were labeled with [^{3}H]thymidine (10 μCi/ml) during the last 8 h of IPTG removal or medium change. Results represent means of quadruple samples ± a standard deviation.

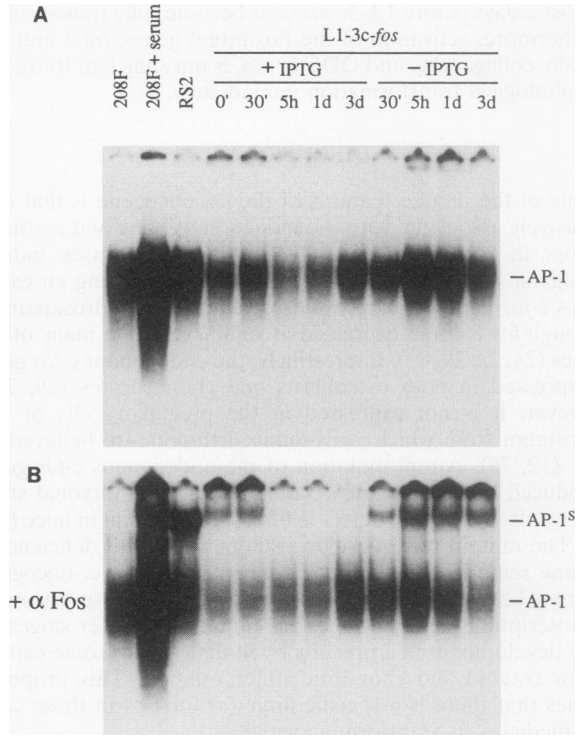


FIG. 8. AP-1 DNA-binding activity in L1-3c-*fos* cells undergoing transformation and reversion. (A) Electrophoretic mobility shift assay of AP-1 DNA-binding complexes in L1-3c-*fos*, 208F, and RS2 cells. Cell extracts (16 μg) used in gel shift assays were prepared from 208F cells cultured in 10% FBS (208F), serum-stimulated (2.5 h) 208F cells (208F + serum), FBJ v-*fos*-transformed 208F cells (RS2), and L1-3c-*fos* cells. L1-3c-*fos* cells were cultured in the presence or absence of IPTG for different lengths of time. The AP-1 probe (25 bp) was derived from the AP-1-binding site in the human metallothionein II_A promoter. (B) Formation of supershifted AP-1 DNA complexes by using the anti-wbFos antiserum (α Fos). The assay conditions are the same as in panel A except that the antiserum (1 μl) was added to the reactions following incubation of the cell extracts with the AP-1 probe. The positions of AP-1 and supershifted AP-1 (AP-1^S) complexes are indicated.

esis (5). Thus, activation of these metalloproteinases may result in alterations in cell-cell and cell-matrix interactions. These changes may contribute, at least in part, to the disorganization of cellular arrangement and the refractile, rounded appearance associated with transformed cells. The activity of matrix metalloproteinases has also been shown to be required, at least in part, for bone resorption (19). Therefore, one possible contributing factor to the observed defect in bone remodeling in mice lacking a functional c-*fos* gene (38, 84) could be a reduction in the activation of collagenase and transin mRNAs by Fos. ODC is known to be a key enzyme involved in the biosynthesis of polyamines that are crucial for cell proliferation (31). Thus, increased expression of ODC mRNA in *fos*-transformed cells could contribute to their accelerated growth rate. Furthermore, it has recently been shown that overexpression of ODC in NIH 3T3 cells leads to cell transformation (2). However, although Fos target genes encoding Fra-1, transin, collagenase, and ODC are rapidly activated following Fos expression, this activation appears to be insufficient to trigger morphological transformation immediately. The observation that cell transformation requires an extended period of Fos expression suggests that at least one of the targets must reach a critical threshold level for full transformation. The delay observed in reversion of transformed cells to a normal phenotype further implies that this protein(s) is relatively long-lived.

Fos, DNA synthesis, and transformation. c-*fos* is a prototypic member of the cellular immediate-early gene family (12). A great number of mitogenic agents induce c-*fos* expression

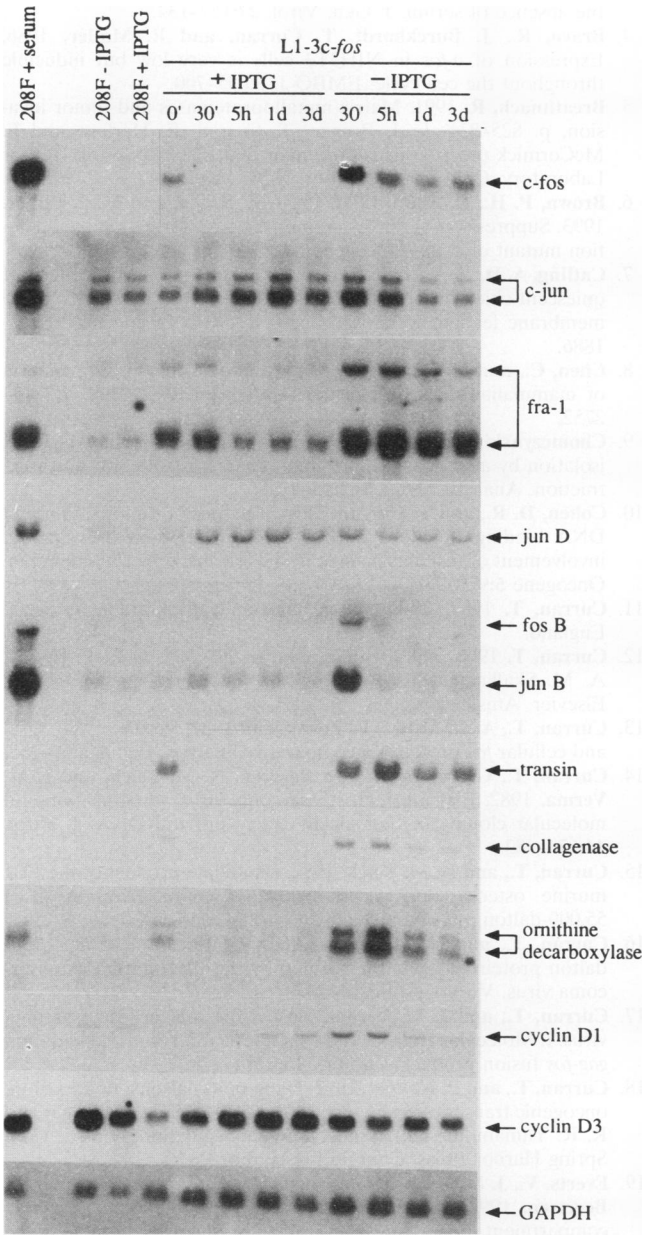


FIG. 9. Northern analysis of potential Fos target genes during *fos*-induced cell transformation and reversion. Cytoplasmic RNA (10 μg) isolated from serum-stimulated (30 min) 208F cells (208F + serum), from normally growing 208F cells in the absence (208F − IPTG) or presence (208F + IPTG) of IPTG, and from L1-3c-*fos* cells cultured in the presence or absence of IPTG for different periods of time was hybridized to various α-^{32}P-labeled cDNA probes as indicated. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. The positions of mRNA are indicated by the arrows.

rapidly and transiently during the G_0-to-G_1 transition prior to the onset of DNA synthesis. These observations led to the proposal that c-*fos* and other immediate-early genes play a role in regulating cell cycle progression. This idea has been extended to suggest that the oncogenic activity of Fos is related to its proposed effects on the cell cycle. However, here we demonstrate that c-Fos induction by itself is unable to stimulate DNA synthesis or cell cycle progression despite the fact that the cells become morphologically transformed (Fig. 7). Furthermore, transformed L1-3c-*fos* cells expressing high levels of c-Fos can be arrested in the G_0 state by serum deprivation, and constitutive expression of c-Fos in these cells had no significant effect on the rate of transition from G_0 to S following serum stimulation (Fig. 6). These observations invite a reassessment of the proposed role of Fos in cell growth.

Attempts to elucidate the role of c-*fos* in cell proliferation through the use of antisense RNA and intracellular injection of anti-Fos antibodies (32, 60, 68) met with mixed success. The antisense RNA approach suffered from substantial variation among results obtained for different cell clones expressing the same antisense RNA. Furthermore, the steady-state levels of antisense RNA expression did not correlate with the effects on cell growth (32, 60). Introduction of Fos antibodies as long as 4 h after serum stimulation into cultured rat fibroblasts was reported to strongly inhibit cell growth (68). However, Fos is present at an extremely low level in fibroblasts 4 h after serum stimulation (Fig. 5B) (4). Subsequent studies demonstrated that microinjection of antibodies against individual members of the Fos family (c-Fos, Fra-1, Fra-2, and FosB) had only a modest effect on cell cycle progression, whereas antibodies cross-reactive with all Fos family members inhibited the G_0-to-S transition efficiently (41, 42). Therefore, it appears that DNA synthesis in serum-stimulated cells does not depend exclusively on the activity of any single Fos protein. This has been underscored by the recent demonstration that embryonal cells, and indeed mice, can survive without a functional Fos gene (20, 38, 84). Thus, while it is likely that Fos-related proteins are involved in some aspects of cell cycle control, Fos does not provide a critical function.

The observation that *fos*-mediated cell transformation occurred in both cycling and noncycling cells indicates that morphological transformation by Fos and cellular proliferation are not linked. The phenomenon that transformation can occur independently of the cell cycle is not unprecedented. For example, oncogenic $p21^{H\text{-}ras}$ is able to induce morphological transformation of quiescent fibroblasts (47, 56). Activated Ras stimulates two independent signal transduction pathways. One leads to DNA synthesis probably through the action of protein kinase cascades, and the other leads to morphological transformation (48). Earlier studies on temperature-sensitive mutants of Rous sarcoma virus also demonstrated that v-*src*-induced transformation can occur in G_0-arrested chicken embryo fibroblasts (3, 49). Thus, distinct signal transduction pathways are utilized in the control of cell transformation and proliferation.

Signal transduction and transformation. Cytoplasmic signal transduction pathways are believed to converge in the nucleus, where they affect the regulation of gene expression. Many products of activated oncogenes have been shown to activate c-*fos* expression. For example, introduction of $p21^{ras}$ into fibroblasts by microinjection rapidly induces c-Fos expression (26, 78). In transient assays, expression of v-*raf*, or v-*src* can activate transcription from the c-*fos* promoter (25, 35). Furthermore, increased AP-1 DNA-binding activity has been observed in cells expressing v-Src (7, 89). These observations have led to the suggestion that c-Fos functions as a nuclear target that mediates the effects of cytoplasmic signaling molecules by regulating gene expression. The use of dominant suppression mutations of Fos and Jun indicates that transformation by *ras*, *raf*, and *src* is dependent on AP-1 proteins (6, 46, 80, 85). Thus, Fos may lie downstream of a common signal transduction pathway, deregulation of which leads to transformation. This implies that cellular transformation by Fos itself

could result from the altered expression of the ultimate target genes of this signaling pathway. However, the results presented here, indicating that there is a significant delay between Fos expression and morphological transformation, are not consistent with this notion.

Previous studies reported that morphological transformation by $p21^{ras}$ occurs within 16 h after introduction of the protein into cells (47, 56, 77). Similarly, cells become fully transformed by oncogenic Raf-1 within 16 h (70). v-*src*-mediated morphological transformation occurs even more rapidly. Temperature-sensitive v-*src* induces cell transformation within several hours after shift to the permissive temperature (24, 69). These events are significantly faster than the period required for Fos to induce transformation. Although partial transformation was observed after 24 h, more than 48 h of Fos expression was necessary for full expression of the transformed cell phenotype (Fig. 3B). Thus, transformation by c-Fos must involve additional target genes to those associated with transformation by *ras*, *raf*, and *src*. Furthermore, while the transforming activities of *ras*, *raf*, and *src* may depend on AP-1 function, c-Fos cannot be fully responsible for this function. In agreement with this notion is the finding that c-Fos is not expressed continuously in *ras*-transformed cells (78). In addition, activation of the c-*fos* gene by *ras* or *src* occurs transiently, lasting for only a few hours (78, 89). Therefore, the effects of transient activation of c-*fos* may differ from those associated with continuous expression that could result in a change in signal transduction pathways.

Fos target(s) for transformation. The delays associated with transformation and reversion indicate that Fos transforms cells by increasing expression of a target gene(s) encoding a long-lived protein that must reach a critical threshold level. This is supported by the observation that lower levels of Fos induce a partially transformed phenotype (Fig. 2). Inhibition of cell division did not reduce the delays required for transformation and reversion. Therefore, the change in cell volume associated with proliferation does not affect the time required for buildup and turnover of the Fos targets. Thus, it is possible that at least one of the targets is an extracellular protein. Since the normal cell morphology is maintained in mixed cultures of normal and Fos-transformed cells, it is possible that this secreted transforming factor is specific for Fos-transformed cells; i.e., these cells could make a receptor or other proteins required for the response to such a factor. Therefore, a network of target genes regulated either directly or indirectly by Fos may participate in the process of *fos*-induced cell transformation.

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